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13. ABSTRACT (Maximum 200 words) <p><b>Background and Purpose:</b> Injury to the central nervous system (CNS) in general, and to the visual system (retina or optic nerve) in particular, has a final outcome which is far more severe than the initial damage. If neighboring neurons that escaped the initial insult are to be rescued from eventual degeneration, ways must be found to protect them. Adequate neuroprotection presupposes a basic understanding of the way in which the damage spreads, the nature of the mediators of toxicity, the most efficient means of neutralizing these harmful agents or their effects, and ways of making the neural tissue more resistant to the toxicity mediators. <b>Methods:</b> Our choice of model for studying mediators of toxicity and methods of neuroprotection is a partial crush injury of the rat optic nerve. <b>Results:</b> (i) Partial injury of the rat optic nerve leads to a gradual spread of damage from the injured neurons, commencing in either the axons or the cell bodies of adjacent neurons that escaped initial injury. (ii) The continuing degeneration is associated with an increase in extracellular glutamate and nitric oxide, and possibly also an increased susceptibility of the spared neurons to the toxicity. (iii) Autoimmune neuroprotection is effective, at least in part, via local production of neurotrophic factors. <b>Conclusion:</b> Partial lesion of the optic nerve results in a self perpetuating and self limiting spread of neuronal damage, which is mediated in part by a toxic increase in physiological compounds, in combination with an increased susceptibility of the affected neurons. Immune neuroprotection is effective, at least in part, by local reactivation of the T cells so as to secrete neurotrophic factors.</p>					
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## 5. Introduction

It is now generally accepted that the final outcome of any acute or chronic injury to the central nervous system (CNS) is far more severe than could be anticipated from the severity of the primary insult. Moreover, the mechanism underlying the spread of damage appears to be common for all CNS injuries, regardless of the site of the insult (optic nerve, spinal cord, brain) or the primary cause (crush, contusion, optic neuropathy, laser retinal injury) (1-9). Prompted by the recognition that the spread of damage after CNS injury is a self-perpetuating process with common mediators, we sought a model that would enable us to follow and demonstrate the effects of such damage (10, 11). An ideal model would allow us to differentiate between the damage caused by the primary insult and by secondary degeneration(11). It would also allow us to elucidate the mechanism(s) underlying the process of damage spread, identify the toxic agent or agent(s) mediating secondary degeneration, and examine the possible existence of mechanisms of self-protection with the object of manipulating them for optimal effectiveness.

Potential strategies for neuroprotective treatment include the elimination of risk factors for nerve damage, neutralizing the toxicity of risk factors (for example, by using glutamate receptor antagonists or inhibitors of nitric oxide synthase), and increasing neuronal resistance to risk factors of exogenous or endogenous origin. Several mediators of toxicity have been identified, among them glutamate, free oxygen radicals, and high  $K^+$ (3,6,12-18). It seems that certain physiological compounds whose normal concentrations are exceeded in response to the damage caused by a primary risk factor may contribute to the progression of degeneration, even after the primary risk factor itself has been alleviated or removed (19-23). If self-emitting physiological compounds (such as glutamate or nitric oxide) at above-normal concentrations are indeed responsible for the progression of neuronal degeneration, ways should be found to neutralize them, compete with them, or increase the resistance of vulnerable neurons to them. This promising neuroprotective approach is now being tested experimentally as a therapeutic strategy in cases of CNS trauma and neurodegenerative diseases.

As mentioned above, the environmental toxicity created by the degenerating nerve causes further neuronal damage. It seems, however, that both the primary and secondary causative factors, whether external (toxicity) or internal (deprivation of target-derived trophic support), not only trigger destructive processes, but also awaken mechanisms of self repair. It appears, though, that in most degenerative diseases of the retina or the optic nerve, these self-repair mechanisms are either too weak or too short-lived to override the destructive processes.

Our studies carried out with the support of the US Army were initially devoted to laser-induced retinal trauma and low-energy laser therapy. Soon after we started, however, we realized that laser-induced trauma and low-energy laser therapy should be studied under the more general heading of injury-induced spread of damage and neuroprotection, respectively. Both require in-depth understanding of the mechanisms underlying the spread of damage after acute injury, and of potential mechanisms to cope with the damage and reinforce them for maximal benefit. We therefore shifted the focus of our studies onto the design of a basic model for assessing the spread of damage and evaluating self-protective mechanisms evoked by the injury. We believe that the outcome of the present study will be of great value in the development of therapies for CNS neuroprotection in general, and not only in the case of traumatic injuries to the optic nerve.

## 6. Body

### I. Establishment of the rat optic nerve as a model for the study of secondary degeneration after white matter injury

#### I.a. *Quantitative proof of neuronal degeneration*

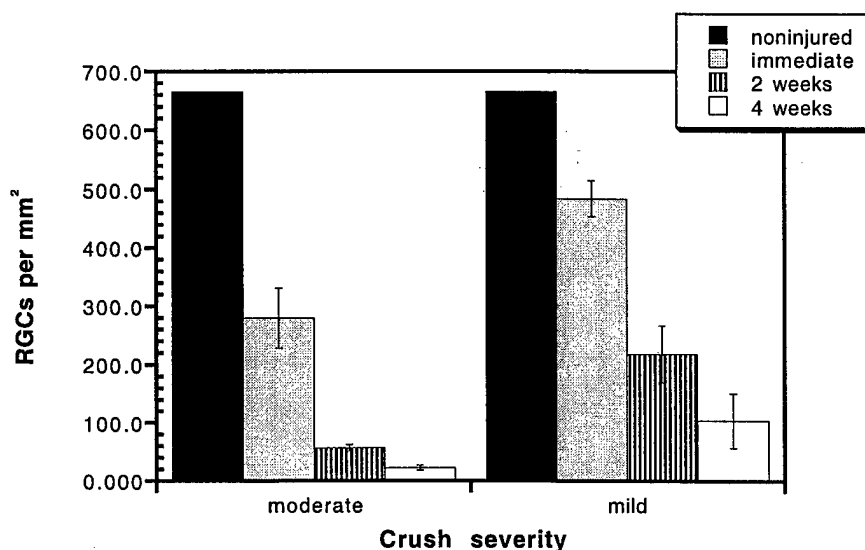
We established a model of a partial crush lesion of the optic nerve of the adult rat. Using this model, we employed a well-controlled procedure that allows quantification of both primary damage and secondary degeneration. This is done by applying a neurotracer dye distally to the site of the lesion immediately (to assess primary damage) and 2 or 4 weeks later (to assess secondary degeneration). To ascertain the number of labeled retinal ganglion cells (RGCs) in each retina, five fields (each of 0.78 mm<sup>2</sup>) were randomly selected and their RGCs counted and averaged. Figure 1 shows the numbers of RGCs per mm<sup>2</sup> in retinas of nerves with

moderate or mild injuries, immediately after the injury and 2 or 4 weeks later. As shown, the extent of primary damage (approximately 58% and 27% in moderate and mild injuries, respectively) is a function of the severity of the insult.

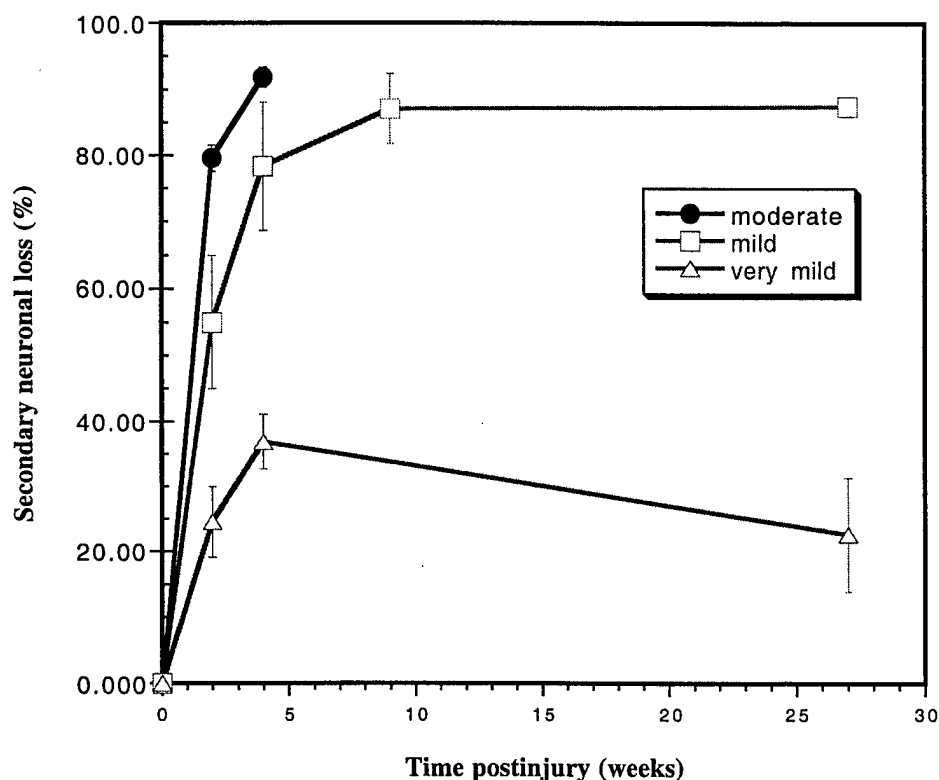
Rats were also examined 9 and 27 weeks after the injury. In these experiments we included some rats whose injuries were very mild. It was interesting to note that the amount of secondary degeneration reflected the severity of the primary insult — the more severe the primary insult, the higher the percentage of secondary degeneration, as calculated by the following equation:

Amount of secondary degeneration (%) =  $[\text{Number of spared neurons (primary - secondary)} / \text{Number of spared primary neurons}] \times 100\%$ .

In both mild and very mild injuries the secondary degeneration was self-limiting, as indicated by the attainment of a steady state (Fig. 2). The reproducibility of the injury is evident from a comparison of six different experiments, each carried out with five to ten rats, in which the percentage survival was determined 2 weeks after the optic nerve crush. The average number of counted RGCs in the six experiments were 50.6, 57.03, 42.5, 42.3, 58.17, and 45.52. The mean value ( $\pm$  SEM) for all the experiments was  $48.85 \pm 3.05$  RGCs. Variations among rats within an experiment were very low, and the results did not differ significantly between experiments.



**Figure 1.** Progressive loss of neurons left intact after axonal injuries of varying severity. The neurotracer dye 4-Di-10-Asp was applied to rat optic nerves at different times after the nerves had been subjected to crush injuries of moderate or mild severity. One week later the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from five fields (located at approximately the same distance from the optic disk) in each retina were counted and their average number per mm<sup>2</sup> was calculated. Bars represent mean values  $\pm$  SEM of retinas of uninjured nerves ( $n = 2$ ); moderately injured nerves that were retrogradely labeled immediately ( $n = 2$ ), 2 weeks ( $n = 6$ ), or 4 weeks ( $n = 8$ ) after injury; and mildly injured nerves retrogradely labeled immediately ( $n = 3$ ), 2 weeks ( $n = 3$ ), or 4 weeks ( $n = 4$ ) after injury.



**Figure 2.** Secondary degeneration of rat optic nerves as a function of time after injuries of varying severity. The figure shows secondary degeneration, defined and calculated as the percentage of neurons that degenerated subsequently to the primary damage. Three groups of injured nerves were included in this comparison: moderately injured, mildly injured, and very mildly injured. Labeling and analysis at the indicated time points after injury were as described in Figure 1. Note that a steady state was reached after mild or very mild injury but not after moderate injury. A minimum of three rats, and in some groups six or eight rats, were examined at all time points.

#### I.b. *Validation of the morphometric method of quantification*

The application of dye distally to the site of the lesion requires a second axotomy. It was therefore important to know whether this second lesion would result in a further loss of labeled RGCs up to the time (7 days later) of retinal excision for RGC counting. If so, we would expect to find fewer labeled RGCs in the retinas of twice-

injured nerves than in retinas of rats subjected only to the first injury. To clarify this point, we carried out the following experiment. Prior to crush injury all RGCs were labeled by stereotactic injection of Fluoro-Gold into the brain. The crush injury was inflicted 2 weeks later. After a further 2 weeks some rats were subjected to an additional axotomy, similar to that performed for the post-crush dye application (as described in Figure 1). Retinas were excised 3 or 7 days later and their RGCs were counted. As shown in Table 1, the second axotomy caused no further significant loss of labeled RGCs. This finding implies that despite the certain death of neurons caused by the second axotomy, it takes more than a week for the dying cell bodies to disappear. This experiment therefore confirms the validity of the morphometric analysis used.

**Table 1.** Number of surviving retinal ganglion cells after crush injury, with or without a second axotomy

Days Post-axotomy	Crush injury No axotomy (RGC/mm <sup>2</sup> ± SEM)	Crush injury + axotomy (RGC/mm <sup>2</sup> ± SEM)
3	1015.5 ± 60.5 (n=2)	974 ± 10.54 (n=3)
7	918 ± 66.5 (n=2)	971.67 ± 38.8 (n=3)

*Note.* In normal retina (with no crush injury or axotomy), there were 2908.5 ± 110.5 RGCs per mm<sup>2</sup>. Fluoro-Gold was injected stereotactically into the superior colliculus of naïve rats. Two weeks later the optic nerves were exposed unilaterally and moderately crushed. After a further 2 weeks, some rats were reanesthetized and the same optic nerves were re-exposed and subjected to a second lesion in the form of a transection distal to the primary insult. Retinas were excised from all rats 3 or 7 days later. Results are expressed as the mean number of RGCs per mm<sup>2</sup> ± SEM. One-way analysis of variance revealed no differences between rats that were subjected to a second axotomy and those that were not ( $p = 0.57$ ).

*I.c. Differential susceptibilities of neurons to the primary insult and to secondary degeneration as a function of their topography*

To find out whether the vulnerability of the axons to primary or secondary degeneration is affected by their topography, we mapped the distribution of RGCs in

retinas of uninjured nerves and of injured nerves 2 and 4 weeks after moderate crush. The distribution patterns in retinas of injured optic nerves are shown in Figure 3. Comparison of the patterns revealed that in the uninjured nerves, RGC density was very similar in all tested zones except for a slight but non-significant tendency to decline towards the retinal periphery (Fig. 4). Following secondary degeneration, this tendency became more pronounced and was significant (Figs. 4 and 5). These findings raised the question of whether topographical selectivity is triggered by the primary insult itself or by subsequent secondary events. To address this question, we analyzed RGC topography immediately after injury. The results showed that immediately after a primary insult, even of mild severity, there is already a preferential loss of neurons whose ganglion cells are located in the retinal periphery (Fig. 6). It therefore seems that the observed topographical selectivity is a reflection of the susceptibility of the axons to the injury, the most vulnerable axons being those whose cell bodies are located peripherally.

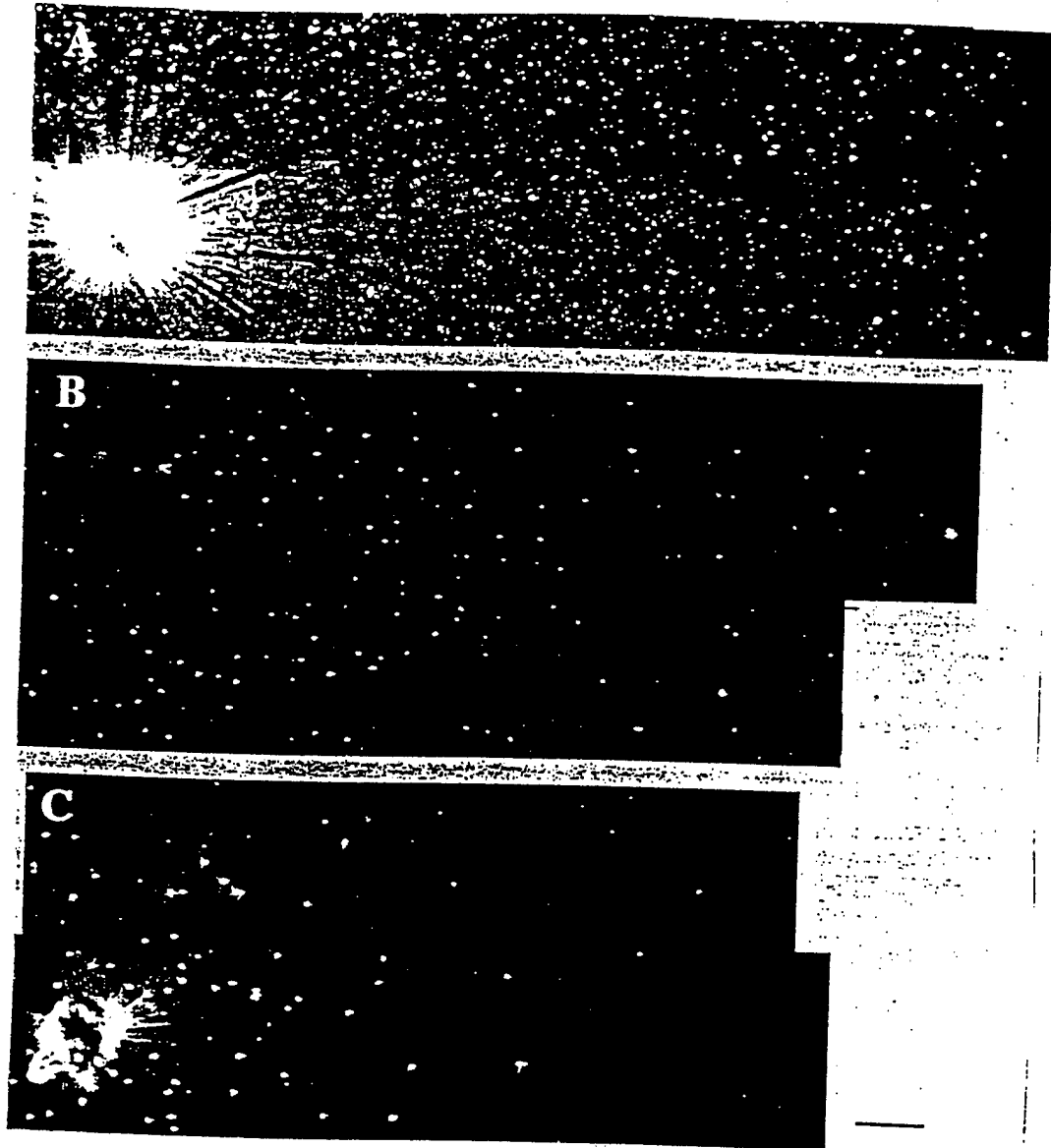
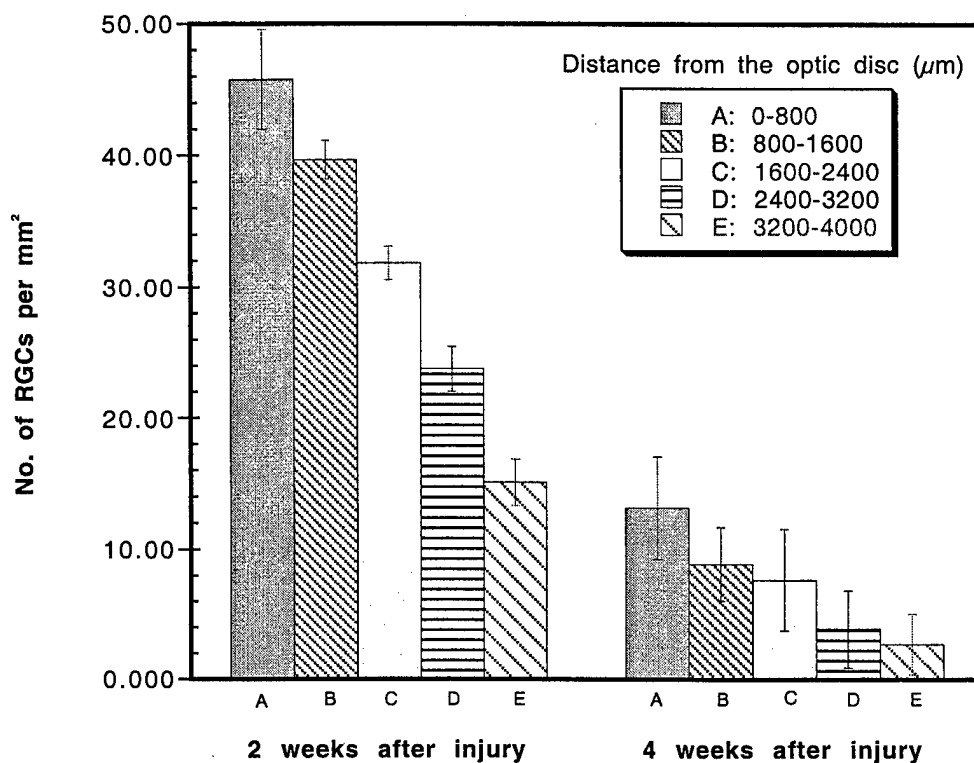
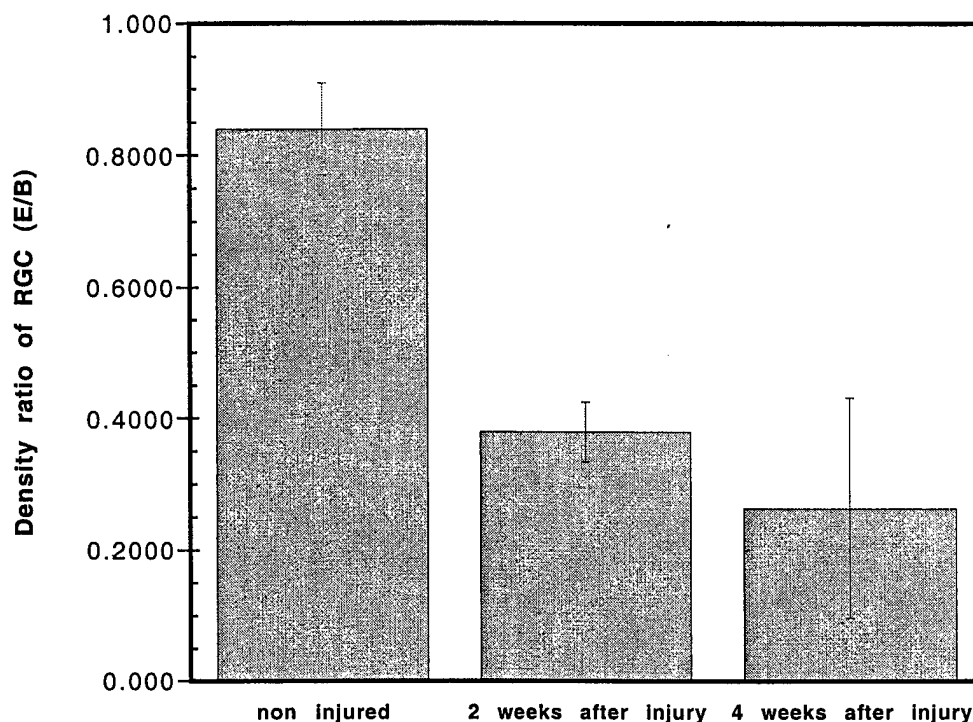


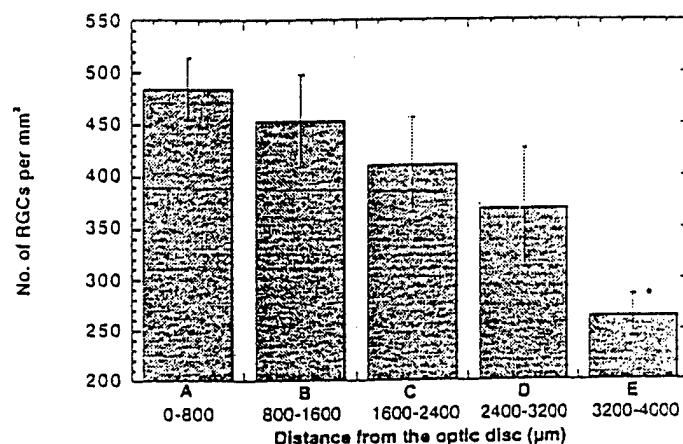
Figure 3. Retrogradely labeled retinal ganglion cells of uninjured nerves after secondary degeneration. The figure shows representative micrographs of normal retina (A), retina of an injured optic nerve labeled 2 weeks after moderate crush injury (B), and retina of an injured optic nerve labeled 4 weeks after moderate crush injury (C). Bar = 300  $\mu$ m.



**Figure 4.** Comparison of retinal ganglion cell distribution in retinas of uninjured nerves and of injured optic nerves 2 or 4 weeks after moderate injury. Retinas were divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated after counting them in four different fields in each zone. The graph shows means  $\pm$  SEM of RGCs in four retinas of injured nerves examined 2 weeks or 4 weeks after injury.



**Figure 5.** Density of retinal ganglion cells at the periphery and in the center of the retina. The distribution of RGCs in normal (uninjured) retina and in retinas of injured nerves 2 weeks or 4 weeks after moderate optic nerve injury was determined by comparing the ratios of cell densities in zone B and zone E in each retina. The zones correspond to those shown in Figure 4. Zone B was chosen rather than zone A because it is closer to the center, and the number of RGCs in this zone does not differ significantly from that in zone A but can be counted more accurately. The graph shows means  $\pm$  SEM of the ratios obtained from two retinas of uninjured optic nerves and from four retinas of optic nerves analyzed 2 weeks and 4 weeks after injury.



**Figure 6.** Primary mechanical insult causes preferential loss of neurons whose ganglion cells are at the retinal periphery. RGCs were retrogradely labeled immediately after mild injury. One week later retinas were excised, whole-mounted, and divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated after counting them in four different fields per zone. The graph shows means  $\pm$  SEM of the numbers of RGCs in four retinas. [ANOVA revealed a significant effect of the distance from the optic disk on the number of RGCs ( $F = 4.28$ ,  $p = 0.028$ ). \*Significant difference ( $p < 0.05$ ) relative to the most central zone (zone A)].

## II. Mediators of cytotoxicity after optic nerve injury

In several species glaucoma have been associated with an elevation of intravitreal glutamate (21, 23). Because the rat eye is small, however, we measured its amino acid content in the aqueous rather than in the vitreous humor, as the absence of viscosity in the aqueous humor allows us, despite its smaller volume, to obtain more accurate measurements than in the vitreous.

Injury of the rat optic nerve was accompanied by some changes in the intraocular content of individual amino acids (21, 23). Examination of the amino acid content in samples of aqueous humor taken 7 days after optic nerve injury and from eyes with uninjured nerves revealed that the excitatory amino acids glutamate and aspartate were the only ones that were significantly increased in amount (24). Glutamate was higher than normal 3 days after injury, was still high on day 7, and returned to normal by day 14. Glutamate concentrations in the uninjured contralateral eyes were similar at the various time periods examined, and differed significantly from those in the injured eyes on days 3 and 7. The absolute concentrations of glutamate and aspartate in the aqueous humor of the injured and uninjured adult rat eye were  $74.25 \pm 14.2 \mu\text{M}$  and  $23.4 \pm 5.1 \mu\text{M}$ , respectively (mean  $\pm$  SEM). The corresponding concentrations 7 days after injury were  $147.6 \pm 15.8 \mu\text{M}$  and  $41 \pm 4.4 \mu\text{M}$ . Glutamate concentrations in the aqueous humor of sham-operated nerves 7 days after surgery did not differ significantly from those in the uninjured eyes. These results rule out any procedure other than the nerve crush injury as the cause of the observed increase in glutamate.

### III. Autoimmunity as a potential mechanism of neuroprotection

Our previous studies related to the role of the immune system in the context of axonal injury in the CNS have indicated that recruitment of T cells to the CNS is relatively limited as compared with peripheral nerves (25). We found, surprisingly, that boosting of the recruitment by passive transfer of T cells, directed against myelin basic protein (MBP), is beneficial and reducing the spread of damage (26). We further found that the neuroprotective effect of the anti-MBP T cells is not correlated with their virulence; T cell line reactive to the whole MBP and the T cell line reactive to the cryptic epitope p51–70 were compared with respect to the severity of the neurodegenerative disease known as experimental autoimmune encephalomyelitis (EAE) they induced, and for their effects on secondary degeneration. In rats injected with the T cell line reactive to the cryptic epitope, disease severity (as manifested by the maximal EAE score) was significantly lower than that in rats injected with the T cell line reactive to the whole protein. Whereas anti-MBP T cells caused clinical paralysis of the limbs, rats injected with the anti-p51–70 T cells developed only tail atony, not hind limb paralysis, and almost none of them showed weakness of the

hind limbs. Despite this difference in EAE severity, the neuroprotective effects of the less virulent (anti-p51-70) and the more virulent (anti-MBP) T cells were similar (26). The percentage of RGCs that survived secondary degeneration was significantly higher in the retinas of rats injected with either of the T cell lines than in the retinas of PBS-injected rats. Thus, there was no correlation between the neuroprotective effect of the autoimmune T cells and their virulence. It is possible that the anti-p51-70 T cells encounter little antigen in the intact CNS, and therefore cause only mild EAE. Their target antigen may however become more available after injury, enabling these T cells to exert a neuroprotective effect.

We further confirmed the neuroprotection by electrophysiological studies. Immediately after optic nerve injury rats were injected i.p. with PBS or with  $1 \times 10^7$  activated anti-MBP or anti-OVA T cells. The optic nerves were excised 7, 11, or 14 days later and their compound action potentials (CAPs), a measure of nerve conduction, were recorded from the uninjured nerves. On day 14, the mean CAP amplitude recorded from the distal segments of injured nerves obtained from rats injected with anti-MBP T cells was about 250% of that recorded from the PBS-injected control rats. As the distal segment of the injured nerve contains both axons that escaped the primary insult and injured axons that have not yet degenerated, the observed neuroprotective effect could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of the injured neurons (which normally occurs in the distal stump), or both. No effect of the injected anti-MBP T cells on the mean CAP amplitudes of uninjured nerves was observed (26). It is unlikely that the neuroprotective effect observed on day 14 could have been due to the regrowth of nerve fibers, as the time period was too short for this.

The strong neuroprotective effect of the anti-MBP T cells seen on day 14 was associated with a significantly decreased CAP amplitude recorded on day 7 (26). The anti-MBP T cells manifested no substantial effect on the uninjured nerve on day 7, indicating that the reduction in electrophysiological activity observed in the injured nerve on day 7 might reflect the larger number of T cells present at the injury site relative to the uninjured nerve (26). The observed reduction in CAP amplitude in the injured nerve on day 7 reflected a transient reduction in conduction, which may have imposed a transient resting state in the injured nerve. This transient effect had

not only disappeared, but was even reversed by day 14 (26). Early signs of the neuroprotective effect could already be detected on day 11 in the rats injected with anti-MBP T cells (data not shown). In rats injected with anti-OVA T cells, no reduction in CAP amplitude on day 7 could be detected in either the injured or the uninjured nerves, and no neuroprotective effect was observed on day 14 (26). Thus, it seems that the early reduction in CAP and the late neuroprotection shown specifically by the anti-MBP T cells are related.

In the course of our studies supported by the US Army we addressed these two issues. We found that a single injection of autoimmune T cells directed against an antigenic epitope within the myelin basic protein leads to a long-lasting effect. The results are summarized in the attached manuscript by Moalem et al. 2000a. In an attempt to get an insight into the possible mechanism underlying the neuroprotection effect of the T cells we examined whether it is a long-lasting effect and whether it is mediated, at least in part, via local production of neurotrophic factors. To this end, we first examined the autoimmune T cells produce neurotrophic factors and whether the secreted levels of the factors depend on reactivation via encountering their specific antigens at the lesion site. We found that T cells are producing a myraid of neurotrophic factros. The levels of the secreted factors is a function of reactivation by their specific antigens. The manuscript summarizing this aspect of the work by Moalem et al., 2000b, supported in part by the US Army, is enclosed in the appendix.

#### IV. Methods

**Animals.** Animal utilization was in accordance with the ARVO resolution on the use of animals in research. Adult male Sprague-Dawley (SPD) rats weighing 300–400 g from the Weizmann Institute of Science animal house were anesthetized with Vetalar (ketamine, 50 mg/kg) and Rompun (xylazine, 0.5 mg/kg) administered i.p. Prior to tissue excision the rats were killed by an overdose of sodium pentobarbitone (170 mg/kg, i.p.).

**Crush injury.** With the aid of a binocular operating microscope, lateral canthotomy was performed in the right eyes of anesthetized rats. The conjunctiva was incised laterally to the cornea, the retractor bulbi muscle was separated and the optic nerve exposed. Using calibrated cross-action forceps, a moderate or mild crush injury (27) was inflicted on the nerve 2 mm from the eyeball, taking special care not to interfere with the retinal blood supply. The use of these forceps makes it possible to inflict a reproducible, controllable injury of different severities by varying the number of screw revolutions attached to the forceps ((11)).

**Morphometric analysis of primary and secondary degeneration.** Primary degeneration was measured by immediate post-injury application of a dye 4-(4-(didecylamino)styryl)-n-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes, Europe BV) distally to the site of injury, since only intact axons are capable of transporting the dye back to their cell bodies. The number of labeled cell bodies is thus a measure of the number of axons that survived the primary degeneration. Secondary degeneration was also measured by application of a dye distally to the injury site, but at various time points after the primary lesion. Such delayed and distal application of the dye ensures that only axons that survived the primary and secondary degeneration will be counted. This approach also makes it possible to differentiate between undamaged neurons and injured neurons with still-viable RGCs, since only those neurons whose fibers are morphologically intact can take up dye applied distally to the lesion site and transport it to their cell bodies. The number of labeled RGCs is thus a reliable reflection of the number of still-functioning neurons.

Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, once again without damaging the retinal blood supply. Solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited 1–2 mm from the distal border of the injury site. Non-injured optic nerves were similarly labeled at approximately the

same distance from the globe. One week after dye application the rat was given a lethal dose of pentobarbitone (170 mg/kg). The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy. RGCs were counted as described in Results. It should be noted that the efficiency of labeling by this procedure is less than 100%, meaning that the method does not yield an absolute number of intact neurons; it does, however, allow a reliable quantitative comparison between treated and untreated nerves. Normalizing the number of fibers relative to that of control noninjured nerves provides a quantitative indicator of the number of intact fibers after injury with and without treatment.

**Collection of aqueous humor.** Rats were deeply anesthetized and their right (injured) and left (non-injured) eyes were excised 2 h or 1, 3, 7, or 14 days after the crush injury, washed quickly three times in PBS, dried carefully, and placed on clean dry Petri dishes. The sclera was punctured with a scalpel and a large incision was made in the sclera and cornea, allowing the fluid to drain out of the eye into the dish. With the aid of a sterile Gilson pipette, fluid specimens were collected, placed in an Eppendorf tube, immediately frozen on dry ice, and maintained at  $-70^{\circ}\text{C}$  until use.

**Glutamate analysis.** Each specimen was dissolved in 100 ml of double-distilled water (DDW) and stirred for 1 min. To separate proteins from the specimens, we ran the specimen through a Sep-Pak column (C-18, Waters Corp., USA) pre-washed with acetonitrile and DDW that were placed on a 45-mm millipore filter. Specimens were dried in the Speedvac and dissolved in 20 ml of DDW. Aliquots (5 ml) of the solution were subjected to amino acid analysis using a reverse phase C18 column (HPL, HP1090), a pre-column derivation procedure, and detection by UV light. To obtain an average, we normalized the results by calculating the amount of each amino acid as a percentage of the total amino acids recovered by the column.

**T cell lines.** T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens. The antigen was dissolved in PBS (1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and draining lymph nodes were surgically removed and dissociated. The cells

were washed and activated with the antigen (10  $\mu\text{g/ml}$ ) in proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100  $\mu\text{g/ml}$ ), non-essential amino acids (1 ml/100) and autologous rat serum 1% (volume/volume). After incubation for 72 h at 37°C, 90% relative humidity and 7% CO<sub>2</sub>, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, and antibiotics, in the same concentrations as above, and also 10% fetal calf serum (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A-stimulated spleen cells. Cells were grown in propagation medium for 4 to 10 days before being restimulated with their antigen (10  $\mu\text{g/ml}$ ) in the presence of irradiated (2000 rad) thymus cells ( $10^7$  cells/ml) in proliferation medium. The T cell lines were expanded by repeated stimulation and propagation.

## 7. Conclusion

1. We have established a rat model that allows us to demonstrate that neurons which have escaped a primary axonal injury will nevertheless degenerate because of their proximity to neurons degenerating as a result of the primary insult.
2. As a result of our work, the occurrence of secondary degeneration after acute or chronic lesions of traumatic or pathogenic origin is now widely recognized as an integral part of the response to any injurious CNS event. We have demonstrated, using the rat optic nerve model, that partial lesion of the optic nerve leads to measurable secondary degeneration. Our findings suggest that it is worthwhile to pursue studies of the role of neuroprotection in cases of RGC and optic nerve degeneration.
3. We have shown that well-known mediators of toxicity, such as glutamate, play a role in RGC degeneration.
4. We show that immune neuroprotection is operating in part via local secretion of neurotrophic factors, though antigen dependent. In the course of our studies of

the crush-injured optic nerve of adult rats, we recently came across another mechanism, traditionally viewed as detrimental, which may be a physiological self-repair mechanisms which is apparently insufficient. The self-repair mechanism in this case operates externally to the optic nerve and is mediated by autoimmune T cells directed against a CNS self antigen. In its normal state, this mechanism appears to be too weak to be effective, but is amenable to exogenous boosting, and potentially lethal to the tissue if it gets out of control. We suggested that the endogenous T cell immune response to optic nerve damage is beneficial, but limited. Our findings confirmed, that exogenous administration of T cells directed against the CNS self antigen MBP significantly reduces the injury-induced spread of degeneration. This boosting of the neuroprotective autoimmunity was achieved without accompanying autoimmune disease, by the adoptive transfer of T cells with selective activity against non-encephalitic self-epitopes. It is conceivable that the endogenous T cells that accumulate spontaneously at sites of CNS injury arise from an injury-triggered autoimmune response. It might therefore be worth seeking ways to augment therapeutically a beneficial autoimmune response without triggering a persisting autoimmune disease. Such boosting might be achieved, for example, by employing T cells specific to the self-antigenic epitopes normally sequestered in the intact CNS. These autoimmune T cells would not accumulate in or interact with undamaged sites, and thus would not induce disease, yet they might be able to assist in the repair of injured CNS tissue if the covert epitope is exposed by the injury.

5. It is conceivable that T cells of different specificities will be employed in the future for neuroprotection against damage to other CNS loci, according to the principles shown to operate in our studies. This possibility is currently being investigated in a model of laser-induced retinal degradation, where the activity of mediators of secondary degeneration and neuroprotection were found to be consistent with our findings in the model of the crush-injured optic nerve.

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**Autoimmune T cells retard the loss of function  
in injured rat optic nerves**

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## **Abstract**

We recently demonstrated that autoimmune T cells protect neurons from secondary degeneration after central nervous system (CNS) axotomy in rats. Here we show, using both morphological and electrophysiological analyses, that the neuroprotection is long-lasting and is manifested functionally. After partial crush injury of the rat optic nerve, systemic injection of autoimmune T cells specific to myelin basic protein significantly diminished the loss of retinal ganglion cells and conducting axons, and significantly retarded the loss of the visual response evoked by light stimulation. These results support our challenge to the traditional concept of autoimmunity as always harmful, and suggest that in certain situations T cell autoimmunity may actually be beneficial. It might be possible to employ T cell intervention to slow down functional loss in the injured CNS.

*Keywords:* Autoimmunity, CNS, injury

## 1. Introduction

Axonal injury in the mammalian central nervous system (CNS) initiates a process of axonal degeneration at the injury site, usually leading to failure of the damaged fibers to regrow and reconnect, and the eventual death of the corresponding cell bodies (Ramon y Cajal, 1959). In addition, fibers that have not sustained direct injury but are located in the vicinity of the injured neurons will, unless adequately treated, undergo secondary degeneration (Faden, 1993; Lynch and Dawson, 1994; Yoles and Schwartz, 1998). This progressive spread of damage results from a process that begins within minutes of injury and continues for days or weeks (Faden, 1993; Yoles and Schwartz, 1998). Among the injury-related mechanisms that might underlie the post-traumatic spread of damage are biochemical and metabolic changes in oxygen and glucose utilization, energy state, lipid-dependent enzymes, free radicals, eicosanoids, tissue ions, biogenic amines, endogenous opioids, and excitatory amino acids (Faden, 1993; Hovda et al., 1991; Liu et al., 1994; Yoles et al., 1992; Yoshino et al., 1991). These changes cause alterations in cellular homeostasis, excitotoxicity, local production of agents harmful to nerve cells, and a loss of trophic support from targets, all of which result in secondary neuronal loss. The proposed mechanisms of secondary degeneration served as a basis for the development and evaluation of various pharmacological interventions for the treatment of CNS injuries. The therapeutic approach of preventing or diminishing the secondary degeneration accompanying CNS trauma is termed neuroprotection (Faden and Salzman, 1992; McIntosh, 1993; Smith et al., 1995).

T cells are important players in the adaptive arm of the immune system. T cells respond to specific antigens through interactions of their specific antigen receptor with the antigen presented by major histocompatibility complex molecules and a group of costimulatory molecules. When activated, they can kill their target cells or produce cytokines that activate or

suppress the growth, movement, or differentiation of other cells. Thus, T cells play a critical part in the protection of tissues against foreign invaders as well as in tissue maintenance.

Immune responses in the CNS are relatively restricted, resulting in the status of the CNS as an immune-privileged site (Streilein, 1995). The unique nature of the communication between the CNS and the immune system can be observed, for example, in the dialog between the CNS and T cells. In the CNS, under normal conditions activated T cells can cross the blood-brain barrier and enter the CNS parenchyma. However, only T cells capable of reacting with a CNS antigen seem to persist there (Hickey et al., 1991). Comparative studies of the T cell response at sites of axotomy in the CNS and the peripheral nervous system (PNS), using T cell immunocytochemistry, revealed a significantly greater accumulation of endogenous T cells in the injured PNS axons than in the injured CNS axons (Moalem et al., 1999b). Moreover, in cases of inflammation, the CNS showed a high potential for elimination of T cells via apoptosis, whereas such elimination was less effective in the PNS, and was almost absent in other tissues such as muscle and skin (Gold et al., 1997). These findings, suggesting that the T cell response to CNS injury is relatively limited, prompted us to examine how augmentation of the T cell response at a site of CNS injury affects the outcome of secondary degeneration.

We recently demonstrated that systemic injection of activated T cells of different antigen specificities immediately after rat optic nerve injury (a model for CNS white matter trauma) results in an increased T cell accumulation at the injury site (Hirschberg et al., 1998; Moalem et al., 1999a). Injection of activated T cells specific to a CNS self antigen, myelin basic protein (MBP), but not to non-CNS antigens, reduced the secondary degeneration of neurons after crush injury of CNS axons (Moalem et al., 1999a). In the present study, we used morphological and electrophysiological techniques to determine the longevity of the induced neuroprotective effect. In addition, measurement of the visual evoked potential (VEP) response, reflecting the functional activity of the visual system in response to a flashing light stimulus (Spekreijse and Apkarian,

1986), was used to determine whether and to what extent the neuroprotective effect is manifested functionally in individual animals over time. We show that autoimmune T cells specific to MBP can slow down the post-traumatic functional loss of the visual response to light in injured optic nerves of adult rats, thereby providing long-lasting neuroprotection.

## **2. Materials and methods**

### *2.1. Animals*

Inbred female Lewis rats (8–12 weeks old) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment.

### *2.2. Antigens*

MBP from the spinal cords of guinea pigs was prepared as described (Hirshfeld et al., 1970). The peptide 277 (p277) of the human 60-kDa heat shock protein (hsp60) (sequence VLGGGCALLRCPALDSLTPANED) (Elias et al., 1991) was synthesized using the 9-fluorenylmethoxycarbonyl technique with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptide was analyzed by HPLC and amino acid composition.

### *2.3. T cell lines*

T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens (Ben Nun et al., 1981). The antigen was dissolved in phosphate-buffered saline (PBS) (1 mg/ml) and emulsified with an equal volume of incomplete

Freund's adjuvant (Difco Laboratories, Detroit, MI) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and the draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), nonessential amino acids (1 ml/100 ml) and autologous serum 1% (volume/volume). After incubation for 72 h at 37 °C, 90% relative humidity and 7% CO<sub>2</sub>, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A-stimulated splenocytes. Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 µg/ml) in the presence of irradiated (2000 rad) thymus cells ( $10^7$  cells/ml) in proliferation medium. The T cell lines were expanded by repeated stimulation and propagation (Ben Nun and Cohen, 1982).

#### 2.4. Crush injury of optic nerve

The optic nerve was subjected to crush injury as previously described (Duvdevani et al., 1990). Briefly, rats were deeply anesthetized by intraperitoneal (i.p.) injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA). Using a binocular operating microscope, lateral canthotomy was performed in the right eye, and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a crush injury 1–2 mm from the

eye. Moderate crush injury was used for short-term trials (2 weeks) and mild crush injury for long-term trials (4 weeks), as these time periods were shown to be optimal for demonstrating secondary degeneration and its response to treatment (Yoles and Schwartz, 1998). The uninjured contralateral nerve was left undisturbed.

### *2.5 Measurement of secondary degeneration by retrograde labeling of retinal ganglion cells*

Secondary degeneration of the optic nerve axons and their attached retinal ganglion cells (RGCs) was measured after post-injury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the lesion site, 4 weeks after crush injury. Because only axons that are intact can transport the dye back to their cell bodies, application of the dye distally to the lesion site after 4 weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach enabled us to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, because only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled RGCs reliably reflects the number of still-functioning neurons. Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Five days after dye application the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy.

## 2.6. *Recording of compound action potential*

Nerves were excised and their compound action potentials (CAPs) recorded in vitro using a suction electrode experimental setup (Yoles et al., 1996). Four weeks after injury and injection of T cells or PBS, rats were killed by i.p. injection of pentobarbitone (170 mg/kg) (CTS Chemical Industries, Israel). Both optic nerves were removed while still attached to the optic chiasma, and immediately transferred to a vial containing a fresh salt solution consisting of NaCl 126 mM, KCl 3 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, NaHCO<sub>3</sub> 26 mM, MgSO<sub>4</sub> 2 mM, CaCl<sub>2</sub> 2 mM, and D-glucose 10 mM, aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at room temperature. After 1 h, CAPs were recorded. In the injured nerve, recordings were obtained from a segment distal to the injury site. This segment presumably contained axons of viable RGCs that had escaped both primary and secondary damage, as well as the distal stumps of non-viable RGCs that had not yet undergone Wallerian degeneration. The nerve ends were connected to two suction Ag–AgCl electrodes immersed in the bathing solution at 37 °C. A stimulating pulse was applied through the electrode, and the CAP was recorded by the distal electrode. A stimulator (SD9, Grass Medical Instruments, Quincy, MA) was used for supramaximal electrical stimulation at a rate of 1 pps to ensure stimulation of all conducting axons in the nerve. The measured signal was transmitted to a microelectrode AC amplifier (model 1800, A-M Systems, Everett, WA). The data were processed using the LabView 2.1.1 data acquisition and management system (National Instruments, Austin, TX). For each nerve, the difference between the peak amplitude and the mean plateau of eight CAPs was computed and was considered as proportional to the number of conducting axons in the optic nerve. The experiments were performed by observers blinded to the treatment received by the rats. In each experiment the data were normalized relative to the mean CAP of the nerves from PBS-injected rats.

## 2.7. *Recording of VEP*

Rats were anesthetized and placed in a small stereotaxis instrument. In the skull of each rat three holes were drilled, through which two electrodes were implanted in the primary visual cortices (V1) and one electrode was implanted in the nasal bone, while the dura was kept intact to minimize cortical damage. The electrodes were gold contact pins (Wire-Pro, Salem, NJ) soldered to stainless steel screws, which were screwed into the holes and cemented to the skull with acrylic cement. The electrode inserted through the hole drilled in the nasal bone was used as a reference point. The other two holes were in area OC1 with coordinates bregma 8 mm and lateral 3 mm. The field potential in each eye, recorded in the contralateral visual cortex, was evoked by stroboscopic light stimulation after implantation of the electrodes. The stroboscopic light had the following characteristics: xenon flash tube (4 W/sec, 1–2 msec duration, 0.3 Hz) amplified 1000 times (AM Systems, microelectrode AC amplifier, model 1800) and digitized (12 bits, 5000 samples per sec) by the use of an NB-MIO 16-9 board (National Instruments) and LabView 2.2.1 data acquisition and analysis software. Before and at different times after a right optic nerve crush injury and concomitant i.p. injection of T cells or PBS, VEP responses were recorded from the two primary visual cortices in each animal. The left visual cortex, which is contralateral to the injured nerve, receives most of its input from the crushed nerve, and the right visual cortex, contralateral to the uninjured nerve, receives most of its input from the intact nerve. During each measurement, the eye on the same side as the cortex from which the response was recorded was covered with black tape to eliminate the minor contribution of that eye to the response. The VEP data were computed and the pattern of the stimulus-specific field potentials (obtained from the average of three recordings, each representing the mean response to 60 light stimuli) was compared to the pattern of the non-stimulus-specific field potential (obtained from the average of three recordings in the absence of a light stimulus) in each case. The field potentials of T cell-injected rats and PBS-injected rats were compared in order to determine the effects of the

treatment on visual system integrity obtained from the left visual cortex contralateral to the injured nerve, and from the right (control) visual cortex contralateral to the intact nerve. The VEP amplitude was calculated as the voltage difference between the first negative peak of the field potential and the subsequent positive peak. Latency was calculated as the time to the first negative peak. The effects of the two treatments on VEP amplitudes and latencies over time were analyzed using the repeated measures analysis of covariance (ANCOVA), including treatment and individual rat (random effect) as nominal effects and day as a covariate. Differences in the relative effects of the two treatments over time were detected by including a treatment  $\times$  day interaction term (i.e., by determining whether the linear effects of day have different slopes for the two treatments). Normality of each data set was achieved by natural logarithmic transformation. The proportion of rats with negative VEP responses in each treatment group was calculated for each time point. Differences between the two treatments over time were examined using logistic regression. Significance was tested using likelihood-ratio Chi square. All analyses were carried out using JMP (SAS Institute 1995, Cary, NC).

### 3. Results

#### *3.1. Autoimmune anti-MBP T cells reduce the loss of RGC survival in injured optic nerves*

We have already demonstrated morphologically that anti-MBP T cells, injected immediately after optic nerve crush injury, protect neurons from secondary degeneration, as measured 2 weeks later (Moalem et al., 1999a). To determine whether this neuroprotective effect can also be seen 4 weeks after injury, rats were injected i.p., immediately after mild optic nerve crush injury, with  $10^7$  activated T cells specific to MBP. As controls, rats were injected either with  $10^7$  activated T cells specific to p277 of hsp60 or with PBS. The anti-MBP T cells induced

the transient monophasic paralytic disease known as experimental autoimmune encephalomyelitis (EAE), which started on day 4 after cell injection, peaked on day 6 and terminated around day 10. Secondary degeneration of the optic nerve axons and their attached RGCs was measured by retrograde labeling 4 weeks after the primary injury and counting the labeled RGCs (reflecting still-viable axons) in each retina. The mean number of surviving RGCs was significantly greater in the retinas of rats injected with anti-MBP T cells than in the retinas of rats injected with anti-p277<sup>1</sup> cells or with PBS (Fig. 1). In contrast, the mean number of surviving RGCs in the retinas of rats injected with anti-p277 T cells did not differ significantly from that in the retinas of PBS-injected rats.

### *3.2. Autoimmune anti-MBP T cells reduce the loss of axonal conduction in injured optic nerves*

To confirm the long-lasting neuroprotective effect of the anti-MBP T cells, we carried out electrophysiological studies using CAP as a measure of nerve conduction. Immediately after mild optic nerve crush injury, rats were injected i.p. with PBS or with  $10^7$  activated anti-MBP T cells. The optic nerves were excised 4 weeks later and CAPs were recorded from the uninjured nerves and from the distal segments of the injured nerves. The mean CAP amplitude recorded from the distal segments of the injured nerves of rats injected with the anti-MBP T cells was significantly greater than that recorded from the PBS-injected control rats (Fig. 2). No effect of the injected anti-MBP T cells on the mean CAP amplitude was observed in uninjured nerves. These results indicate that autoimmune anti-MBP T cells produce long-term neuroprotection after CNS axonal injury.

### *3.3. Loss of VEP response to light in injured optic nerves is retarded after administration of anti-MBP T cells*

For functional analysis of the effect of anti-MBP T cells on optic nerve degeneration, rats with electrodes implanted at the visual cortex were injected i.p., immediately after moderate unilateral optic nerve crush injury (right side), with PBS or with  $10^7$  activated anti-MBP T cells. VEP responses to light, previously shown to be a reliable measure of the integrity of the visual system from the retina to the cortex (Dorfman et al., 1987; Spekreijse and Apkarian, 1986), were recorded before injury and 1, 5, 7, and 15 days after injury, from both injured and uninjured nerves of rats injected with anti-MBP T cells or with PBS. The VEP responses were recorded during repetitive visual stimulation (flashing light) and showed separate components corresponding to electrical activity at each relay of the visual pathway from the eye to the cortex. The results were computed and analyzed for the potentials evoked by the left visual cortex receiving information mainly from the injured nerve and the right visual cortex (control) receiving information mainly from the uninjured nerve, before and at different time points after injury. Representative VEP responses recorded from the left visual cortex, before and after optic nerve injury in a rat injected with anti-MBP T cells or with PBS, are shown in Fig. 3.

In rats with positive VEP responses the latencies and amplitudes were analyzed, and the percentage of rats with negative VEP responses in each group was calculated and analyzed separately. A significantly slower decline of VEP amplitude over time, manifested by a less steep slope, was observed in the injured nerves of rats injected with anti-MBP T cells than in the injured nerves of rats injected with PBS (Fig. 4A), indicating that the autoimmune anti-MBP T cells slow down the loss of the visual response to light. In contrast, there was no significant difference in VEP amplitude over time between the uninjured nerves of the two groups of rats (Fig. 4B). The latency of the response peaks did not differ significantly either in the injured or in the uninjured nerves of the rats injected with anti-MBP T cells compared with the PBS-injected

rats (Fig. 4C, D). For both treatments, the probability of a negative VEP response increased over time. However, the percentage of rats that showed negative VEP responses from the injured nerve was higher in PBS-injected rats (78% on day 15) than in rats injected with anti-MBP T cells (50% on day 15), though the difference was not significant (Fig. 4E). No negative VEP responses were obtained from the uninjured nerves in either of the groups. These results indicate that the autoimmune anti-MBP T cells retard the loss of function in injured rat optic nerves.

#### **4. Discussion**

The CNS is an immune-privileged site, in which immune activity is rather restricted (Streilein, 1995). Since immune responses are essential for tissue protection and repair, immune privilege might limit the ability of the CNS to defend itself against trauma. Whereas damage to peripheral tissues is followed by functional recovery, damage to CNS tissue is followed by the degeneration of directly damaged neurons, as well as the progressive secondary degeneration of neurons that escaped the primary injury (Faden, 1993; Ramon y Cajal, 1959; Yoles and Schwartz, 1998). We recently showed that systemic administration of activated T cells specific to a self-antigen, MBP, is beneficial in reducing the spread of damage following CNS axotomy in rats (Moalem et al., 1999a). In the present study we demonstrated that the neuroprotective effect exerted by the autoimmune anti-MBP T cells is long-lasting, being detectable for at least 1 month after optic nerve crush injury in the rat, and is manifested both morphologically and electrophysiologically. The integrity of the visual system was significantly less affected by secondary degeneration in rats injected with anti-MBP T cells than in control PBS-injected rats, thus demonstrating a functional neuroprotective effect of autoimmune anti-MBP T cells in the injured rat optic nerve.

We used three independent and complementary approaches — morphological measurement, and electrophysiological recording of CAP and of VEP — to study the effect of autoimmune T cells on the outcome of optic nerve injury in the rat. The morphological result was achieved by direct counting of retrogradely labeled RGCs, yielding the number of axons that were still viable. The CAP assessment is a direct in vitro measurement of the propagative properties of a bundle of axons; a higher CAP amplitude signifies a larger number of conducting axons (Stys et al., 1991). The CAP is recorded from a segment of the injured nerve that contains both axons that escaped the primary insult and injured axons that have not yet degenerated. Therefore, a higher CAP amplitude could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of injured axons, or both. In this study, the results of the CAP measurement verified the morphological analysis showing that the neuroprotective effect of the autoimmune anti-MBP T cells lasts for at least 4 weeks after optic nerve injury.

Unlike the morphological and CAP measurements, which are performed at a single time point in each rat, measurement of VEP enables us to carry out a non-invasive follow-up of the same rat over time. The VEP does not measure directly the number of RGCs or the number of axons in the optic nerve, but assesses the overall activity of cortical neurons in response to a flashing light stimulus (Dorfman et al., 1987; Spekreijse and Apkarian, 1986). The potentials evoked by the light originate in the retina and are propagated along the axons to reach their final target, the visual cortex. The visual activity is affected by crush injury of the optic nerve and the consequent secondary degeneration. Only axons that have survived both the primary and the secondary degenerative processes are capable of conducting action potentials. Thus, a comparison of the VEP amplitude between the rats injected with T cells and the PBS-injected rats reveals the effect of treatment on the integrity of the visual pathway as a whole. The smaller decline in VEP amplitude observed in rats subjected to optic nerve crush injury and injection of anti-MBP T cells than in control PBS-injected rats indicated that the visual system of the T cell-

treated rats was much less affected by the secondary degeneration. The neuroprotective effect of the anti-MBP T cells on RGC survival and on the number of conducting axons after optic nerve injury was indeed manifested in an improved overall functional activity of the visual system.

These results further substantiate the notion that autoimmunity can be beneficial (Cohen, 1992), at least in CNS injuries, and suggest that although potentially pathogenic, autoimmune T cells can help to maintain tissue homeostasis and repair following trauma. It is possible that the autoimmune response triggered by injury is beneficial and is tightly regulated by self-tolerance mechanisms, leading to autoimmune disease only when its regulation is impaired. Pathological autoimmune diseases develop in only 3% of the population, despite the presence of self-reactive lymphocyte populations in all individuals (Burns et al., 1983; Martin et al., 1990; Pette et al., 1990; Schluesener and Wekerle, 1985). Mechanisms that terminate immune responses are important in self tolerance, where lymphocytes capable of recognizing self antigens are generated constantly, yet normal individuals remain unresponsive to their own antigens (Van Parijs and Abbas, 1998). Recent studies have demonstrated, however, that autoimmunity is awakened in response to CNS injury; T cells isolated from rats subjected to contusive injury of the spinal cord are capable of inducing EAE when transferred to naïve animals (Popovich et al., 1996). The spontaneous T cell response does not, however, exert enough protection to cause significant improvement after CNS injury. This might be attributable to immune privilege, which accounts for the inefficient and restricted communication between the CNS and the immune system. However, appropriate modulation of the immune responses at a site of CNS injury can promote protection and recovery of the CNS. We previously demonstrated that the neuroprotection induced by autoimmune T cells is similar whether the T cells are strongly or weakly encephalitogenic (Moalem et al., 1999a). Thus, the therapeutic use of non-encephalitogenic autoimmune T cells in the injured CNS might prove effective in reducing secondary damage, and thereby preserving neuronal function.

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## Figure legends

**Fig. 1.** Anti-MBP T cells diminish the loss of RGCs after optic nerve injury. Immediately after mild optic nerve injury rats were injected with anti-MBP T cells, anti-p277 T cells, or PBS. Four weeks after injury, the neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the injury site. Five days after dye application the retinas were excised and flat-mounted. Labeled RGCs from five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy, and their average number per mm<sup>2</sup> was calculated. The histogram shows the mean number of RGCs  $\pm$  SE. Each group contained six to ten rats. The neuroprotective effect of the anti-MBP T cells compared with that of PBS or of the anti-p277 T cells was significant (\*\*,  $p < 0.01$ , one-way ANOVA followed by Bonferroni's multiple comparison t-test).

**Fig. 2.** Anti-MBP T cells diminish the loss of CAP in injured optic nerves. Immediately after mild optic nerve injury, rats were injected with either PBS or activated anti-MBP T cells. Four weeks later the CAPs of injured and uninjured nerves were recorded. Results were normalized by calculating the ratio between the mean CAP amplitude of nerves from T cell-injected rats and from PBS-injected rats. The histogram shows the normalized mean CAP amplitudes (%)  $\pm$  SE. Each group contained six rats. There were no significant differences in mean CAP amplitudes between uninjured nerves obtained from PBS-injected rats and from T cell-injected rats. However, the neuroprotective effect of the anti-MBP T cells (relative to PBS) on the injured nerve on day 28 after injury was significant (\*,  $p < 0.05$ , Student's t-test).

**Fig. 3.** Representative VEP responses to light after optic nerve injury. Immediately after moderate optic nerve injury, rats with electrodes implanted in the visual cortex were injected i.p.

with PBS or with activated anti-MBP T cells ( $T_{MBP}$ ). VEP responses to light were recorded before and 1, 5, 7, and 15 days after injury, from both injured and uninjured nerves. The figure shows representative VEP responses recorded from injured optic nerves of rats injected with T cells or with PBS. The gray line represents the VEP response in the absence of a light stimulus. For clarity, it is shown only at the VEP response recorded before the optic nerve injury. Note the reduction in VEP amplitude over time, and the differences in VEP responses between a rat injected with T cells and a PBS-injected rat on days 7 and 15 after injury.

**Fig. 4.** Anti-MBP T cells slow down the loss of VEP response to light after optic nerve injury. Immediately after moderate optic nerve injury, rats with electrodes implanted in the visual cortex were injected i.p. with PBS or with activated anti-MBP T cells ( $T_{MBP}$ ). VEP responses to light were recorded before and 1, 5, 7, and 15 days after injury, from both injured and uninjured nerves. The computed results were analyzed for the effects of the two treatments on VEP amplitudes and latencies over time and for the probability of negative VEP responses over time. Each group contained nine or ten rats. **(A)** VEP amplitudes (natural logarithmic transformed) from the visual cortices contralateral to the injured nerves. The slope of VEP amplitude over time in the anti-MBP T cell treatment was significantly less steep than that obtained with PBS treatment ( $p = 0.029$ , ANCOVA). **(B)** VEP amplitudes (natural logarithmic transformed) from the visual cortices contralateral to the uninjured nerves. There was no significant difference in VEP amplitude over time between the two treatments ( $p = 0.702$ , ANCOVA). **(C)** VEP latencies (natural logarithmic transformed) from the visual cortices contralateral to the injured nerves. There was no significant difference in VEP latency over time between the two treatments ( $p = 0.482$ , ANCOVA). **(D)** VEP latencies (natural logarithmic transformed) from the visual cortices contralateral to the uninjured nerves. There was no significant difference in VEP latency over time between the two treatments ( $p = 0.803$ , ANCOVA). **(E)** Probability of negative VEP

responses. There was no significant difference in recorded negative VEP responses between the two treatments ( $p = 0.3$ , Chi square test). Notably, however, there was a higher proportion of negative VEP responses in the PBS-treated rats than in the rats treated with anti-MBP T cells.

Fig. 1

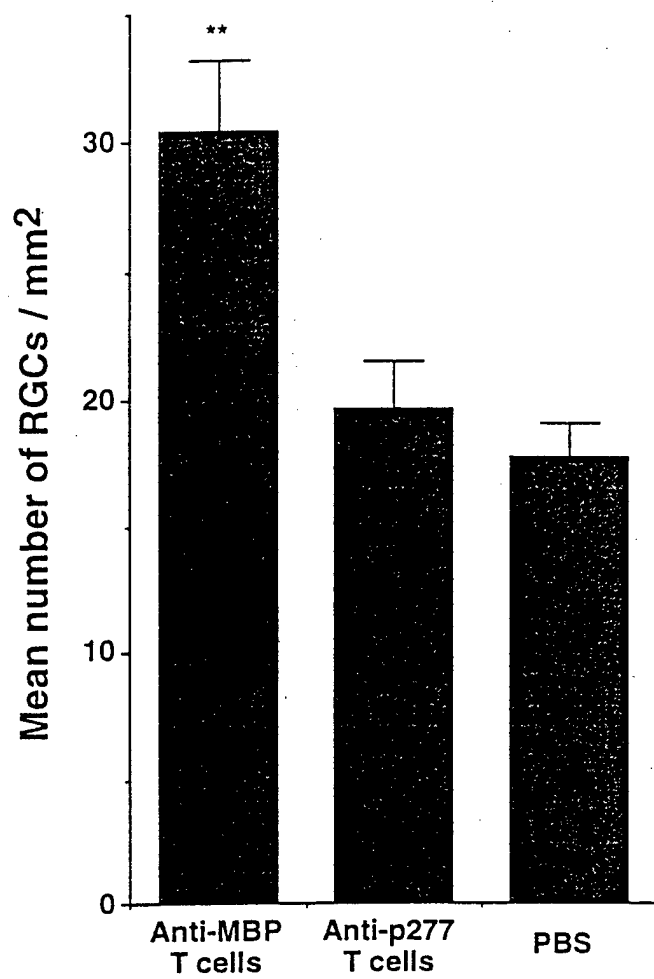


Fig. 2

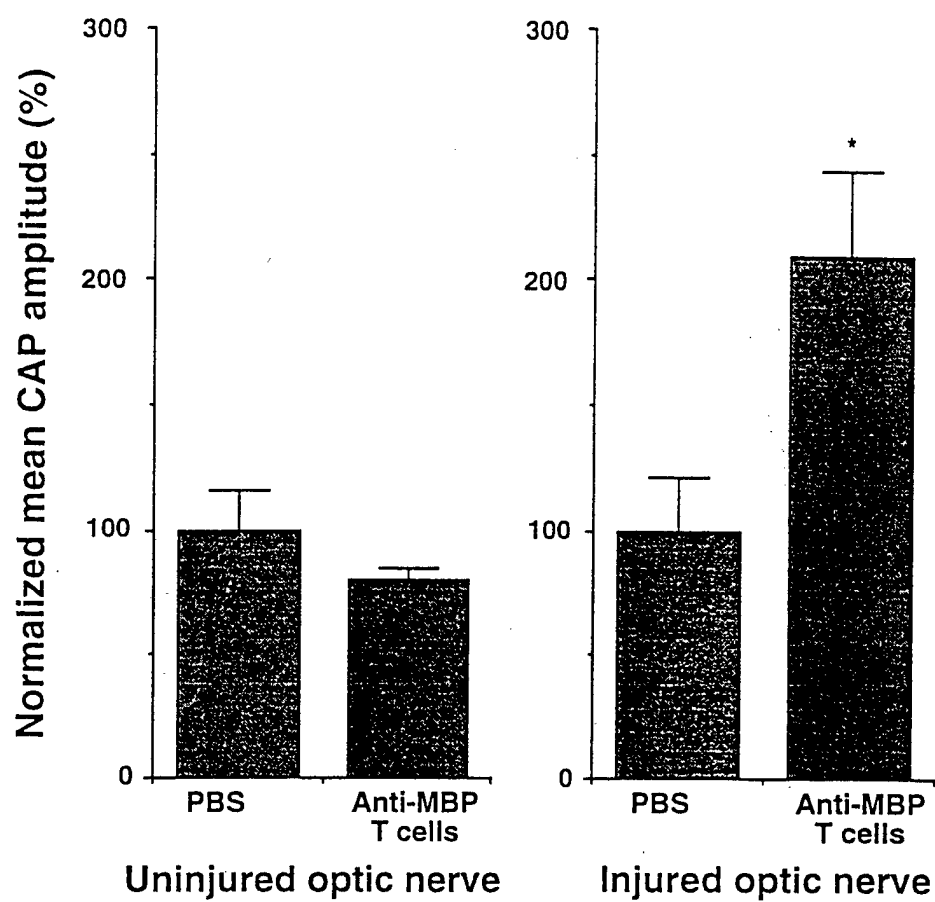


Fig. 3

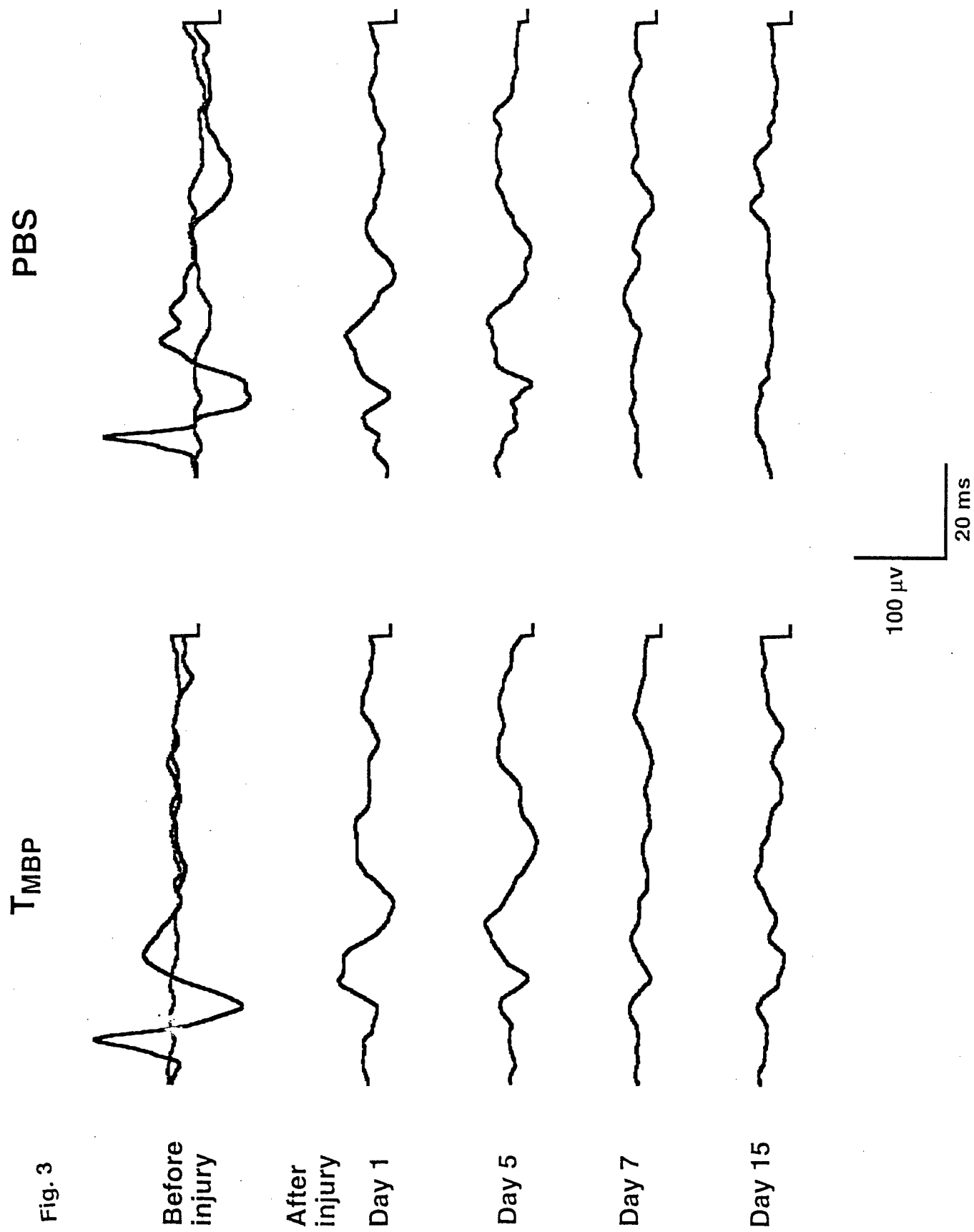
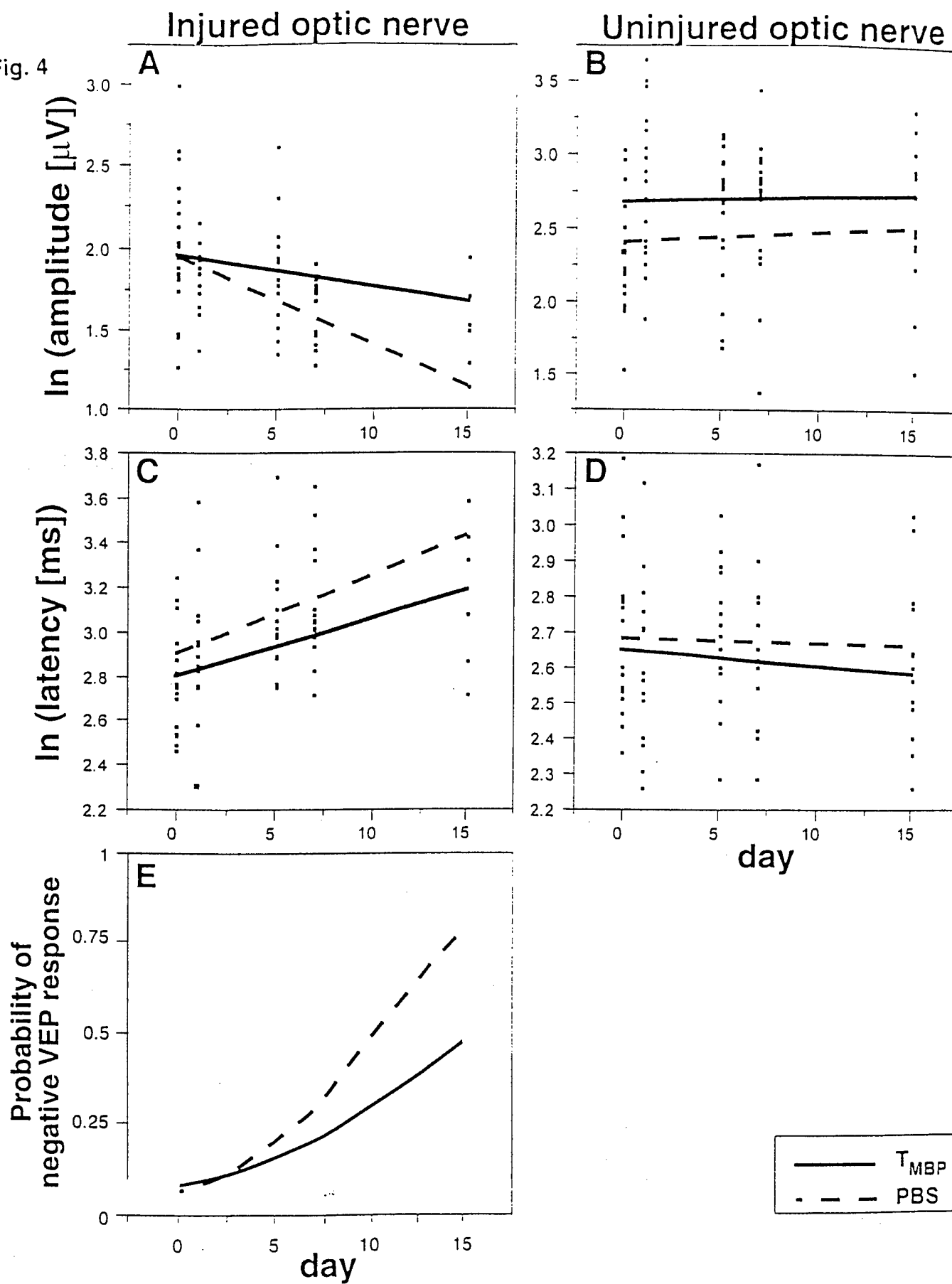


Fig. 4



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Cellular/Molecular Neuroscience

## **Production of Neurotrophins by Activated T Cells: Implications for Neuroprotective Autoimmunity**

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*Abbreviated title:* Neuroprotective autoimmunity involves T cell neurotrophins

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## ABSTRACT

Neurotrophins (NTs) promote neuronal survival and maintenance during development and after injury. However, their role in the communication between the nervous system and the immune system is not yet clear. We recently observed that activated T cells of various antigen specificities home to injured central nervous system (CNS), yet only autoimmune T cells specific to a CNS antigen, myelin basic protein (MBP), protect neurons from secondary degeneration after crush injury of the rat optic nerve. Here we examined the involvement of NTs in the T cell-mediated neuroprotection, and the possible significance of the antigen specificity of the T cells. Analysis of NT expression in various rat T cell lines showed that the T cells express mRNA and protein specific to nerve growth factor, brain-derived neurotrophic factor, NT-3, and NT-4/5. Antigen activation significantly increased NT secretion. Thus, it appears that only CNS autoimmune T cells, upon recognizing their antigen, can secrete increased amounts of NTs in injured optic nerves. mRNA for TrkA, TrkB and p75 receptors was expressed in the injured nerve, suggesting that these specific receptors can mediate the effects of the T cell-derived NTs. The neuroprotective effect of the autoimmune anti-MBP T cells in injured optic nerves was significantly decreased after local application of a tyrosine kinase inhibitor known to be associated with NT-receptor activity. These results suggest that the neuroprotective effect of autoimmune T cells is mediated, at least in part, by their ability to secrete NTs upon antigen activation in the injured CNS. T cell intervention in the traumatized CNS might prove a useful means of promoting post-injury CNS maintenance and recovery via NT supply.

*Key words: neuroprotection; CNS; injury; neurotrophic factors; Trk; lymphocytes; experimental autoimmune encephalomyelitis*

## INTRODUCTION

Neurotrophins (NTs) are biologically active proteins that promote neuronal survival through receptor-mediated processes and are thought to participate in nervous system development, maintenance, and response to trauma. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 have all been shown to enhance neuronal survival *in vitro* and *in vivo*. For example, NGF is required for the survival of sympathetic and some sensory and cholinergic neuronal populations (Levi Montalcini, 1987), BDNF prevents the death of motoneurons in newborn rats after nerve transection (Sendtner et al., 1992) and rescues spinal cord motoneurons from axotomy-induced cell death (Yan et al., 1992), and NT-4/5 increases adult rat retinal ganglion cell (RGC) survival and neurite outgrowth (Cohen et al., 1994; Sawai et al., 1996). The responsiveness of target cells to a given NT is governed by the expression of two classes of NT receptors: the low-affinity receptor p75, which binds all NTs with similar affinity (Chao, 1994), and the high-affinity tyrosine kinase receptors of the Trk family, which interact with NTs in a specific manner. TrkA is the receptor for NGF, TrkB is the receptor for BDNF and NT-4/5, and TrkC is the main receptor for NT-3 (Barbacid, 1994; McDonald and Chao, 1995). Specific binding of the NT to its receptor causes receptor dimerization and autophosphorylation. These processes can activate multiple signal transduction pathways depending on the cell type (Kaplan and Stephens, 1994), leading to different biological functions.

Accumulating evidence suggests that the NTs, in addition to their neurotrophic effects, participate in the inflammatory response. Recently, NGF and the NGF receptor TrkA were shown to be expressed in activated CD4-positive T cell clones (Ehrhard et al., 1993; Santambrogio et al., 1994). NGF is also expressed in B cells (Torcia et al., 1996), in macrophages and microglia (Elkabes et al., 1996; Heese et al., 1998), in mast cells (Leon et al., 1994), in eosinophils (Solomon et al., 1998), and in basophils (Burgi et al., 1996). NT mRNA is expressed

in spleen and thymus (Laurenzi et al., 1994) and in inflamed tissue (Oddiah et al., 1998). Recent studies have shown that activated human T cells, B cells, and monocytes produce BDNF in vitro and in inflammatory brain lesions (Kerschensteiner et al., 1999), and that human stimulated peripheral blood mononuclear cells express TrkB and TrkC receptors (Besser and Wank, 1999). The production of NTs by immune cells provides a novel example of the commonality of the nervous and the immune systems. Some NTs have been shown to modulate certain functions of the immune system. For example, NGF was recently shown to influence lymphocyte proliferation and differentiation (Thorpe and Perez Polo, 1987; Otten et al., 1989), stimulate immunoglobulin production (Otten et al., 1989), and induce mast cell degranulation (Mazurek et al., 1986). Both NT-3 and BDNF induce proliferation and phagocytic activity of microglia (Elkabes et al., 1996).

We recently demonstrated that although the central nervous system (CNS) is a site of immune privilege in which immune responses are normally limited, it can nevertheless benefit from immune activity for its protection and recovery after injury. More specifically, we showed that the T cell response to axonal injury is much more limited in the CNS than in the peripheral nervous system (PNS); significantly more endogenous T cells were found to accumulate in injured PNS axons than in injured CNS axons (Moalem et al., 1999b). Moreover, elimination of T cells via apoptosis occurred extensively in the injured CNS, but only to a very small extent in the injured PNS (Moalem et al., 1999b). Systemic injection of activated T cells of different antigen specificities immediately after CNS injury resulted in an increased accumulation of T cells at the injury site (Hirschberg et al., 1998; Moalem et al., 1999a). Notably, injection of activated T cells specific to a CNS self-antigen myelin basic protein (MBP), but not to non-CNS antigens, reduced the secondary degeneration of neurons after a primary crush injury of CNS axons (Moalem et al., 1999a).

The mechanism underlying this neuroprotective effect of autoimmune anti-MBP T cells in the injured CNS has not yet been established. However, several lines of evidence implicate cytokines and neurotrophic factors as mediators in the reciprocal relationship between the immune and nervous systems. The therapeutic application of NTs prevents neuronal degeneration after axotomy and other forms of neuronal injury (Semkova and Kriegstein, 1999). In addition, beneficial effects of NTs have been reported in animal models of neurodegenerative disease (Mitsumoto et al., 1994). We were therefore interested in determining whether the T cells that confer neuroprotection after CNS axonal injury do so by providing a natural source of imported NTs.

In the present study we show that rat T cells produce various NTs and secrete them upon antigen activation. The optic nerve was found to express TrkA, TrkB and p75 receptors. The neuroprotective effect mediated by autoimmune T cells in injured optic nerve appears to involve T cell NTs, as shown by the fact that local application of K252a, an inhibitor of tyrosine kinase known to be associated with NT-receptor signaling (Koizumi et al., 1988; Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992), partially prevented this neuroprotective effect. These findings suggest that T cell autoimmunity specific to CNS myelin antigens, under certain circumstances, can be beneficial in CNS trauma via NT supply.

## **MATERIALS AND METHODS**

*Animals.* Inbred female adult Lewis rats (8–12 weeks old) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment. Animals were handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee).

*Antigens.* MBP from the spinal cords of guinea pigs was prepared as described (Hirshfeld et al., 1970). Ovalbumin (OVA) was purchased from Sigma (St. Louis, MO). Peptide 277 (p277) of the human 60-kDa heat shock protein (hsp60) (sequence VLGGGCALLRCPALDSLTPANED) (Elias et al., 1991) was synthesized by the 9-fluorenylmethoxycarbonyl technique using an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptide was analyzed by HPLC and amino acid composition.

*Antibodies.* Rabbit polyclonal antibodies raised against amino acids 1–20 of rat NGF, amino acids 128–147 of rat BDNF, and amino acids 139–158 of rat NT-3 were purchased from Santa Cruz (Santa Cruz, CA). Mouse monoclonal anti rat T cell receptor (TCR) antibody (Hunig et al., 1989) was kindly provided by Dr. Boris Reizis. Rhodamine goat anti rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum proteins), and R-phycoerythrin (R-PE) donkey anti rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA).

*T cell lines.* T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens (Ben Nun et al., 1981). The antigen was dissolved in phosphate-buffered saline (PBS) (1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and the draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in stimulation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), nonessential amino acids (1 ml/100 ml), and autologous serum 1% (volume/volume). After incubation for 72 h at 37 °C, 90% relative humidity and 7% CO<sub>2</sub>, the

cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (FCS) (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A (ConA)-stimulated spleen cells (Gillis et al., 1978). Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 µg/ml) in the presence of irradiated (2000 rad) thymus cells ( $10^7$  cells/ml) in stimulation medium. The T cell lines were expanded by repeated stimulation and propagation (Ben Nun and Cohen, 1982).

*Crush injury of optic nerve.* Crush injury of the optic nerve was performed as previously described (Duvdevani et al., 1990). Briefly, rats were deeply anesthetized by intraperitoneal (i.p.) injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA). Using a binocular operating microscope, lateral canthotomy was performed in the right eye, and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a moderate crush injury 1–2 mm from the eye. The uninjured contralateral nerve was left undisturbed.

*Immunocytochemistry.* Activated T cells were separated from irradiated thymus cells using a Ficoll gradient (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation for 20 min at 2000 rpm. The interphase was collected and washed three times in PBS. The blood-derived T cells were purified using a Uni-SepMaxi+ kit (Novamed, Jerusalem, Israel), which is based on density separation of lymphocytes. This was followed by passage through a nylon wool column (Novamed), exploiting the property of B cells, but not T cells, to adhere to nylon wool. The cells were fixed with ethanol for 10 min, washed three times with PBS, permeabilized with acetone for 3 min, washed twice with PBS, and resuspended in PBS containing 3% FCS and

2% bovine serum albumin. The cells were then incubated for 1.5 h at 37 °C with the primary antibody. For the neutralization assay, the antigen-blocking peptide (Santa Cruz) was added to the sample at a peptide:antibody rate of 5:1 (by weight), according to the manufacturer's instructions. The cells were washed three times in PBS containing 0.05% polyoxyethylene sorbitan-monolaurate (Tween-20), then incubated with the second antibody for 1 h at room temperature. The cells were again washed three times with PBS containing 0.05% Tween-20, and then analyzed by flow cytometric (FACS) analysis or viewed with a Zeiss Universal fluorescence microscope. In the latter case, the cells were treated with glycerol containing 1,4-diazobicyclo-(2,2,2)octane to inhibit quenching of fluorescence.

*FACS analysis.* The immunostained cells were resuspended in 0.4 ml of PBS and analyzed by FACScan, with at least 1700 events scored. In single-color analysis, positive cells were defined as cells with greater immunofluorescence, on a logarithmic scale, than that of control cells incubated with second antibody only, or with second antibody in the presence of the non-corresponding first antibody as a cross test. The cells were scored from a region defined according to physical parameters that indicate the size (forward scatter) and granularity (side scatter) of lymphocytes.

*Reverse transcriptase-polymerase chain reaction.* To examine the expression of NTs and cytokines, activated rat T cells were separated from irradiated thymus cells as described above, and total RNA was extracted from the activated T cells in each cell line using the commercial TRI reagent (Molecular Research Center, Cincinnati, OH). To examine NT receptor expression, total RNA was isolated from rat optic nerves 7 days after injury, as well as from normal nerves (using the TRI reagent, Molecular Research Center), according to the manufacturer's instructions. First-strand cDNA synthesis reaction was carried out as follows: 1 µg of RNA and 3.5 mM oligo (dT) primer were incubated at 65 °C for 5 min and then chilled on ice, after which 0.5 mM dNTP mixture, AMV-RT (Promega, Madison, WI), and reverse transcriptase (RT) buffer were added,

according to the manufacturer's instructions. The mixture was incubated at 42 °C for 1 h, then at 94 °C for 2 min, and placed on ice. From the generated complementary DNA, a volume of 2–4 µl was amplified in a total volume of 50 µl, with 2.5 U of taq-zol (Tal-Ron, Rehovot, Israel), 0.2 mM dNTP mixture, and 50 pmoles of each primer. The primer sequences are listed in Table 1. For the polymerase chain reaction (PCR) amplification, we used the thermocycler Cetus with the following settings: 94 °C for 3 min for RNA denaturation, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 1 min at 60 °C, and elongation for 2 min at 72 °C. The amplified products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A band of each NT and Trk receptor was purified from the gel using the GenElute<sup>TM</sup> kit (Supleco, Bellefonte, PA), and sequenced to confirm its specificity.

*Enzyme-linked immunosorbent assay.* Anti-MBP T cells were grown for a week in a propagation medium, then washed with PBS and resuspended in stimulation medium. The T cells ( $0.5 \times 10^6$  cells/ml) were incubated, in the presence of irradiated thymocytes ( $10^7$  cells/ml), with ConA (1.25 µg/ml), or with MBP antigen (10 µg/ml), or with OVA antigen (10 µg/ml), or with no antigen, in stimulation medium at 37 °C, 90% relative humidity and 7% CO<sub>2</sub>. In addition, irradiated thymocytes ( $10^7$  cells/ml) alone were incubated in stimulation medium. After 48 h the cells were centrifuged and their supernatants were collected and sampled. Concentrations of NT-3, NGF, and NT-4/5 in the samples were determined by the use of sandwich enzyme-linked immunosorbent assay (ELISA) kits (Promega, Madison, WI) and comparison with an NT standard (absorbance measurement at 450 nm using an ELISA reader). BDNF concentrations in the samples were determined with a sensitive sandwich ELISA. In brief, 96-well flat-bottomed plates were coated with a chicken anti-human BDNF antibody (Promega, Madison, WI) in 0.025 M NaHCO<sub>3</sub> and 0.025 M Na<sub>2</sub>CO<sub>3</sub> (pH 8.2). Recombinant human BDNF (used as standard; Research Diagnostics, Flanders, NJ) was used in serial dilutions in blocking solution containing 3% bovine serum albumin, 0.05% Tween-20, and 1% FCS in PBS (pH 8.2). Bound BDNF was

detected by incubating the plates with a mouse anti-human BDNF antibody (Research Diagnostics) followed by peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) in blocking solution. The plates were developed using a 3,3',5,5'-tetramethyl-benzidine liquid substrate system (Sigma, St. Louis, MO). The reaction was stopped by adding 1M  $H_3PO_4$ , and the optical density was determined at 450 nm. Results for each experiment were calculated as the amount of secreted NT per 1 ml of sample, after subtraction of the background levels of the irradiated thymocytes incubated with the stimulation medium.

*Measurement of secondary degeneration by retrograde labeling of retinal ganglion cells.*

Secondary degeneration of the optic nerve axons and their attached RGCs was measured after post-injury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the site of injury, 2 weeks after the primary injury. Only axons that are intact are capable of transporting the dye back to their cell bodies. Therefore, application of the neurotracer dye distally to the site of the primary crush after 2 weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, because only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled RGCs reliably reflects the number of still-functioning neurons. Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Five days after dye application the rats were killed. The retina was detached from the

eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy.

*Administration of K252 compounds.* Sterile stock solutions (10 mM) of K252a and K252b were prepared in dimethyl sulfoxide, stored at  $-20^{\circ}\text{C}$ , and diluted in PBS before use. One day after right optic nerve injury and injection of T cells or PBS, each rat received one drop of 1  $\mu\text{M}$  K252a, K252b, or vehicle in the right eye, three times a day for 13 days. On day 14, the neurotracer dye 4-Di-10-Asp was applied to the optic nerves distally to the site of injury, and 5 days later the retinas were examined for labeled RGCs (reflecting still-viable axons).

*Clinical evaluation of experimental autoimmune encephalomyelitis.* To examine for the presence and severity of experimental autoimmune encephalomyelitis (EAE), rats were scored every 1 to 2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

## RESULTS

### **mRNA for cytokines of Th1, Th2 and Th3 phenotypes, and for the NTs NGF, BDNF, NT-3, and NT-4/5, is expressed in rat T cell lines**

Because passive transfer of T cells specific to a CNS self antigen MBP, but not of T cells specific to the non-CNS antigens p277 or OVA, exerts a neuroprotective effect (Moalem et al., 1999a), we examined whether these T cell lines differ in their cytokine mRNA profiles and whether they transcribe NT mRNA. The anti-MBP, anti-p277 and anti-OVA T cell lines were activated with their respective antigens for 2 days and total RNA was then extracted from the lymphoblasts of each line. Qualitative RT-PCR was then carried out, using primers specific for the various cytokines and NTs. The three tested T cell lines, anti-MBP ( $T_{\text{MBP}}$ ) (Fig. 1A), anti-

p277 ( $T_{p277}$ ) (Fig. 1B), and anti-OVA ( $T_{OVA}$ ) (Fig. 1C), expressed cytokines of Th1 ( $IFN\gamma$ , and IL-2 which is not shown), Th2 (IL-10 and IL-6), and Th3 ( $TGF\beta$ ) subsets. In addition, all three T cell lines expressed NT-3, NGF, NT-4/5 and BDNF mRNA. The PCR product of each NT was sequenced and its identity confirmed. A PCR control reaction for each NT and cytokine, containing all the components except the complementary DNA, was negative.

### Activated rat T cells produce NT proteins

To determine whether the NT mRNA is translated into a corresponding protein in the activated T cells, we performed immunocytochemistry for NTs. Activated rat anti-MBP, anti-p277 and anti-OVA T cells were immunostained with rabbit antibodies directed against rat NGF, BDNF or NT-3, detected by rhodamine goat anti-rabbit IgG. As shown in Figure 2, the T cells expressed NGF, BDNF and NT-3 immunoreactive proteins. The cells were viewed and photographed with a fluorescence microscope using a filter that detects rhodamine (Fig. 2, left), and using phase contrast (Fig. 2, right). Staining in the absence of the first antibody was negative (-) (Fig. 2). Binding of the first antibody was neutralized with the corresponding blocking peptide, yielding negative staining.

To examine the incidence of NT-producing cells in the T cell lines, we used FACS analysis. Activated anti-MBP, anti-p277 and anti-OVA T cells were immunostained for BDNF, NT-3, or NGF, and in each case also for TCR. The FACS analysis is presented in Figure 3A and the definition of quadrants in Figure 3B. FL<sub>1</sub> indicates FITC staining for TCR and FL<sub>2</sub> indicates R-PE staining for the specific NT. Regardless of antigen specificity, in each T cell line almost all of the cells that were stained for TCR (right side of each plot) were also stained for NT (upper right of each plot) (Fig. 3A). These results indicate that the three tested T cell lines produce NTs, and that the incidence of T cells expressing NTs is high and is similar in all three lines.

### **Blood-derived T cells express intracellular NT proteins**

The T cell lines were cultured by repeated stimulations and propagations in vitro. To determine whether non-manipulated T cells also express NTs, we isolated T cells from the blood of naïve Lewis rats. One day later we subjected the cells to immunostaining for BDNF, NT-3, or NGF and in each case also for TCR, and then subjected them to analysis by FACS. As shown in Figure 4, naïve blood-derived T cells were stained for NGF, BDNF, and NT-3. The percentage of T cells that were stained for NTs (upper right of each plot in double staining) was higher than 80%, suggesting that the high prevalence of intracellular NT expression in T cells is not exclusive to particular T cell lines. To examine the specificity of the staining, binding of the first antibodies to their corresponding blocking peptides was performed. Neutralization of the anti-NT antibodies by addition of the blocking peptides led to a marked decrease in staining (Fig. 4), indicating that the observed staining is specific.

### **Secretion of NT proteins by T cells is significantly augmented upon activation**

Next we examined whether the T cells also secrete NTs, and if so, whether the NT secretion depends on antigen activation. T cells can be activated by polyclonal mitogens or by their specific antigen. Therefore, supernatants were collected after 48 h from activated anti-MBP T cells that were incubated with the polyclonal mitogen ConA, or with their specific antigen MBP, and from non-activated anti-MBP T cells that were incubated with a non-specific OVA antigen or with no antigen. The supernatants were then subjected to sensitive sandwich ELISA. The NTs are known to function physiologically at extremely low (picomolar) concentrations. The activated T cells secreted NGF, NT-3, NT-4/5 and BDNF proteins (Fig. 5). However, significantly higher levels of each of these proteins were secreted by the activated anti-MBP T cells than by the non-activated anti-MBP T cells, indicating that NT secretion by T cells is induced by activation. The non-specific antigen (OVA) induced no more NT secretion than that

obtained by the anti-MBP T cells alone. This finding suggests that unlike anti-p277 or anti-OVA T cells, autoimmune anti-MBP T cells can be activated to secrete high levels of NTs in the injured optic nerve, presumably because they are the only ones capable of recognizing their antigen there.

#### **mRNA for TrkA, TrkB, and p75 receptors is expressed in the optic nerve**

To express their physiological effects, the NTs should recognize their specific tyrosine kinase receptors of the Trk family and/or the low-affinity p75 receptor (Segal and Greenberg, 1996). In a previous study we demonstrated that autoimmune T cells exert a neuroprotective effect in rat injured optic nerves (Moalem et al., 1999a). We were therefore interested in determining whether mRNA for Trk or p75 receptors is expressed in the optic nerve, a potential site of interaction with T cell-derived NTs. Total RNA was extracted from normal optic nerves and from injured optic nerves 7 days after moderate crush injury. The RNA was then subjected to qualitative RT-PCR, using primers specific for TrkA, TrkB, TrkC, and the p75 receptor. Both the normal and the injured optic nerves were found to express mRNA for TrkA, TrkB, and p75 receptors, but not for the TrkC receptor (Fig. 6). A positive control PCR for TrkC receptor was performed on total RNA isolated from rat brain (data not shown). For each NT receptor we performed a negative control PCR, in which all the components except the complementary DNA were present. The PCR product of TrkA and TrkB receptors was sequenced and its identity confirmed. The finding that mRNA for TrkA, TrkB and p75 receptors is expressed in the optic nerve points to the ability of T cell NGF, BDNF, NT-4/5, and possibly also NT-3 to interact with their receptors and mediate their biological effects on neuronal survival in the injured optic nerve.

### **Blockade of signaling associated with NT receptors reduces the neuroprotective effect of the autoimmune anti-MBP T cells in the injured optic nerve**

K252a and K252b are microbial alkaloids that block NT receptor signaling by preventing autophosphorylation of the tyrosine kinase domain of the Trk receptors (Koizumi et al., 1988; Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992). Both K252a and K252b are hydrophobic, but K252a is irreversibly concentrated in intracellular membranes and is thus more potent than K252b (Ross et al., 1995). To determine whether blocking of signal transduction associated with NT activity influences the neuroprotective effect of autoimmune anti-MBP T cells at and near the optic nerve lesion site, we performed the following experiment. Immediately after undergoing moderate unilateral optic nerve crush injury (right side), rats were injected i.p. with  $10^7$  activated anti-MBP T cells or with PBS, and a drop of 1  $\mu$ M K252a, K252b, or vehicle was instilled in the right eye, three times a day, from day 1 after injury until day 14. On day 14, RGCs were retrogradely labeled and the mean numbers of surviving RGCs with intact axons were calculated. In addition, the rats were scored every 1–2 days for clinical EAE. Neither the course of EAE nor the disease severity (Fig. 7A) was affected by the daily eye-drop treatment with K252a or K252b, eliminating the possibility of any systemic influence of K252 on T cell infiltration into the CNS. After daily treatment with vehicle eye drops, the mean numbers of surviving RGCs were significantly greater in the retinas of rats injected with anti-MBP T cells than in rats injected with PBS, demonstrating the neuroprotective effect of the autoimmune T cells. However, the mean numbers of surviving RGCs were significantly lower in the retinas of rats injected with anti-MBP T cells and treated daily with K252a eye drops than in those injected with anti-MBP T cells and treated daily with the vehicle eye drops (Fig. 7B). No significant effect of K252b eye drops on the mean numbers of surviving RGCs was observed in the rats injected with anti-MBP T cells. Likewise, no effect of K252a or K252b eye drops on the mean numbers of surviving RGCs was observed in PBS-injected rats (Fig. 7B), indicating that the inhibitory effect of K252a

in the rats injected with anti-MBP T cells is associated with the T cell NTs. This finding shows that local blockade of NT receptor signaling reduces RGC survival in the injured optic nerves of rats injected with anti-MBP T cells, and supports the contention that NTs mediate, at least in part, the neuroprotective effect of the autoimmune T cells.

## DISCUSSION

NTs are essential for neuronal survival and maintenance during the processes of development and maturation (Levi Montalcini, 1987; Nitta et al., 1993) and for the regulation of neurotransmitter release and dendritic growth (Thoenen, 1995; Lewin and Barde, 1996; Barde, 1997). Several studies have shown that administered NTs can rescue injured or degenerating neurons and induce axonal outgrowth and regeneration (Sendtner et al., 1992; Yan et al., 1992; Gravel et al., 1997; Kobayashi et al., 1997; McTigue et al., 1998). While NTs have been intensively studied in the nervous system, little is known about their role in the interaction between the nervous and the immune systems. We showed here that rat T cells express mRNA and protein of NGF, BDNF, NT-3, and NT-4/5 and that secretion of the NT proteins by the T cells is significantly increased by antigen activation. We also demonstrated that the neuroprotective effect of the autoimmune T cells is attributable, at least in part, to their ability to supply the injured CNS with NTs.

NTs bind to and activate specific receptor tyrosine kinases of the Trk family (Barbacid, 1994; McDonald and Chao, 1995; Segal and Greenberg, 1996). The interaction of NGF with TrkA receptor, and of BDNF and NT-4/5 with TrkB receptor is rather specific. However, although NT-3 primarily activates TrkC receptor (Lamballe et al., 1991), it can induce moderate activation of TrkA (Cordon-Cardo et al., 1991) and TrkB (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991) receptors, at least in certain cell culture systems (Ip et al., 1993). In addition to binding to a specific Trk, each of these four NTs (NGF, BDNF, NT-3, and NT-4/5) interacts

with a common receptor, the low-affinity p75 receptor (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992). This receptor is primarily expressed on NT-responsive cells and has been shown to play a role in apoptosis (Rabizadeh et al., 1993; Casaccia-Bonofil et al., 1996) and cell migration (Anton et al., 1994). Despite its ability to interact specifically with each of the NTs, p75 appears to be neither necessary nor sufficient for many aspects of NT signaling. Nevertheless, the presence of p75 may modulate the cellular response to NTs (Barker, 1998). For example, the presence of p75 appears to enhance the sensitivity of the response of TrkA to NGF (Davies et al., 1993; Verdi et al., 1994). Our finding that TrkA, TrkB and p75 receptors are expressed in the injured optic nerve supports the possibility that T cell-derived NGF, NT-4/5, BDNF, and perhaps NT-3 can mediate a neuroprotective action at the lesion site. Local blockade of NT-receptor signaling by K252a indeed caused a marked attenuation of the neuroprotective effect of the autoimmune anti-MBP T cells in injured optic nerves, confirming that — at least in part — NT secretion by the autoimmune T cells is responsible for their neuroprotective effect.

Although K252a is a non-selective inhibitor of tyrosine kinase activity, at low concentrations it specifically inhibits Trk receptors and blocks TrkA, TrkB, and TrkC with approximately equal efficacy, while leaving intact other tyrosine kinase signaling pathways, as well as the protein kinase C pathway (Koizumi et al., 1988; Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992). Since the Trk receptors can be expressed in axons and/or glial cells of the optic nerve (Condorelli et al., 1995), the T cell-derived NTs may act directly on the RGCs or on glial cells that can indirectly affect neuronal survival. For example, T cell-derived NTs may locally activate microglia, which in turn release a second NT (Fallon and Loughlin, 1993). Regardless of whether the NT is released directly by the T cells or indirectly by the glial cells, after the NT binds its receptor, the NT-receptor complex is then internalized and transported retrogradely in the axon to the cell body, where it initiates signal transduction (Fallon and

Loughlin, 1993). Thus, it is conceivable that K252a, applied locally as eye drops in this study, acted at the RGCs, blocking the signal transduction of the Trk receptors in response to NTs.

We have previously demonstrated that autoimmune anti-MBP T cells, but not anti-p277 or anti-OVA T cells, exert a neuroprotective effect in rat injured optic nerves (Moalem et al., 1999a). Regardless of their different antigen specificities, these T cell lines did not differ in the expression of their NT profiles *in vitro*. The *in vivo* neuroprotection mediated by the autoimmune T cells and not by the other tested T cell lines can be partly explained, however, in terms of NT secretion from the CNS-specific T cells following interaction of the T cells with their target self antigen presented at the lesion site. We showed here that secretion of NTs by the T cells depends on antigen activation. Thus, the exposure of myelin at the site of injury might activate MBP-specific T cells to secrete NTs that increase neuronal survival, whereas T cells specific to other antigens might not be activated because of inadequate antigen recognition. Signaling via the TCR might therefore be required for NT secretion, allowing only CNS-specific T cells to exert a neuroprotective effect after axonal injury. NT release by T cells can directly or indirectly increase neuronal survival through mechanisms such as the regulation and buffering of ion concentrations, or the control of  $\text{Ca}^{2+}$  levels by modulating calcium-binding proteins, enzymes, and membrane  $\text{Ca}^{2+}$  channels. Whether a single NT or a combination of different NTs is responsible for the neuroprotective effect of the autoimmune T cells is still an open question. Future experiments should include specific blocking of each NT, so that their specific contribution to the T cell-mediated neuroprotection can be evaluated.

Intensive research over the last few decades has been aimed at uncovering the reasons why the CNS fails to accomplish processes of protection, repair, and regeneration. A growing body of evidence indicates that under normal physiological conditions the interaction between the immune system and the CNS is restricted (Schwartz et al., 1999), and that this restriction strongly influences the events which follow CNS injury (Perry et al., 1987; Moalem et al.,

1999b). Effective dialog between any injured tissue and the immune system is, however, a prerequisite for healing. Circumvention of the restricted communication between the CNS and macrophages by transplantation of activated macrophages into the CNS lesion site in the rat optic nerve or spinal cord results in regrowth and partial recovery (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998). Systemic administration of activated T cells specific to a self antigen, MBP, is beneficial in reducing the spread of damage following CNS axotomy in rats (Moalem et al., 1999a). We have suggested that autoimmunity, unlike autoimmune disease, is a benign immune response that protects the CNS against pathogen-free damage. In the present study we demonstrate that the autoimmune neuroprotection exhibited after injury by anti-MBP T cells can be attributed, at least in part, to NT activity derived from autoimmune T cells.

Our results suggest that, although potentially pathogenic, autoimmune T cells can help to maintain tissue homeostasis and repair after trauma. Autoimmunity can be benign (Cohen, 1992), and may thus represent a beneficial physiological response evoked by the injury to protect the tissue from the spread of damage. The fact that the spontaneous response does not exert enough protection to cause significant improvement after CNS injury might be attributable to the restricted and/or inefficient communication between the CNS and the immune system. T cell accumulation at a site of CNS axonal insult is significantly increased after injury and passive transfer of activated autoimmune T cells (Hirschberg et al., 1998; Moalem et al., 1999a), but the phenotype of the neuroprotective T cells is not yet known. We showed here that the injected anti-MBP T cells express cytokines of Th1, Th2 and Th3 subtypes *in vitro*. It is not yet possible, however, to determine whether the neuroprotective effect seen *in vivo* is produced by all of the injected cells or only by a certain subpopulation of them. We are currently investigating the phenotype of the T cells isolated from the injury site.

In conclusion, we demonstrate that rat T cells produce NTs and secrete them upon activation. We suggest that the beneficial effect of autoimmune T cells on injured CNS axons is

exerted via local secretion of NTs by the T cells in response to activation by their specific antigens. Thus, NTs may play an important role in the communication between the nervous and the immune systems. Withdrawal of growth factors is one of the mechanisms leading to death of neurons in CNS injuries and neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (Semkova and Krieglstein, 1999). It might be possible to prevent this neuronal cell loss by a direct delivery of neurotrophic factors. An obstacle to the development of effective therapy, however, is delivery of NTs to the CNS, because these proteins are not able to cross the blood-brain barrier.

Systemic injection of activated autoimmune T cells specific to a CNS antigen appears to be a feasible cell therapy that offers some advantages: first, these T cells can cross the blood-brain barrier (Hickey et al., 1991) and specifically accumulate at the site of CNS lesions (Hirschberg et al., 1998), and secondly, the T cells are capable of continuously releasing various NTs at the CNS injury site, and the timing and dynamics of such NT release might be in accordance with the needs of the tissue. This procedure may therefore potentially offer a physiological form of CNS maintenance that might be worth developing as a therapeutic modality for CNS injuries if the autoimmune responses are properly controlled. In other words, neuroprotection — without risk of autoimmune disease — might be achieved by the use of autoimmune T cells specific for a CNS antigen, which do not induce an autoimmune disease but do accumulate at the site of CNS injury, provide NTs, and protect neurons from secondary degeneration.

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## FIGURE LEGENDS

**Figure 1.** Expression of mRNA for cytokines and NTs in activated rat T cell lines. Anti-MBP T cells ( $T_{MBP}$ ), anti-p277 T cells ( $T_{p277}$ ), and anti-OVA T cells ( $T_{OVA}$ ) were activated with their respective antigens for 2 days. Total RNA was extracted from the lymphoblasts, and qualitative RT-PCR was performed using primers specific for various cytokines and NTs.  $T_{MBP}$  (A),  $T_{p277}$  (B) and  $T_{OVA}$  (C) cells expressed mRNA for cytokines of Th1 (IFN $\gamma$ ), Th2 (IL-10 and IL-6), and Th3 (TGF $\beta$ ) subsets, as well as for the NTs NT-3, NGF, NT-4/5, and BDNF.

**Figure 2.** Detection of NTs in the T cells by immunocytochemistry. The rat T cell lines  $T_{MBP}$ ,  $T_{p277}$ , and  $T_{OVA}$  were activated with their respective antigens for 3 days, and then immunostained with rabbit antibodies directed against rat NGF, BDNF, or NT-3, detected by rhodamine goat anti rabbit IgG. The cells were viewed and photographed with a fluorescence microscope, using a filter that detects rhodamine (left), and using phase contrast (right). Staining in the absence of the first antibody (–) was negative.

**Figure 3.** Flow cytometric analysis for the expression of NTs by activated T cells. **A.** Activated rat  $T_{MBP}$ ,  $T_{p277}$ , and  $T_{OVA}$  cells were immunostained for BDNF, NT-3, or NGF, and in each case also for TCR, and then analyzed by FACS. FL<sub>1</sub> indicates FITC staining for TCR, and FL<sub>2</sub> indicates R-PE staining for the specific NT. About 70% of the cells were double-stained. Irrespective of their antigen specificity, almost all cells that showed staining for TCR (right side of each plot) also showed staining for NT (upper right of each plot). **B.** Definitions of quadrants. To define the cell populations that showed staining for NT or for TCR, a single staining was done for each of them. The populations were then defined according to the positive and negative controls. For each single staining the positive control, with the corresponding first and second

antibodies (Abs), is shown on the left. The negative controls were cells stained with second antibody only (middle), and as a cross test, cells stained with the corresponding second antibody, in the presence of the non-corresponding first antibody (right). Because of overlapping of the TCR-stained and TCR-unstained populations, they were distinguished using the histogram (bottom).

**Figure 4.** Flow cytometric analysis of blood-derived T cells for expression of NTs. T cells were derived from rat blood by passage through a Percoll gradient and then through a nylon wool column. The cells were immunostained for NTs and TCR and then analyzed by FACS. FL<sub>1</sub> indicates FITC staining for TCR and FL<sub>2</sub> indicates PE staining for the specific NT. About 50% of the cells were double-stained. A high proportion of the cells that were stained for TCR (right side of each plot) also showed staining for NT (upper right of each plot). For each NT a neutralization assay was carried out, using the commercial blocking peptides of the first antibodies (Abs), to confirm their specificity. The results can be seen by comparing the plots on the left side (double staining) with those on the right (neutralization of anti-NT Ab).

**Figure 5.** ELISA of secreted NTs. Rat anti-MBP T cells were cultured for 48 h in stimulation medium with ConA, MBP, OVA, or no antigen. The supernatants of the T cells were collected and subjected to sandwich ELISA. The histogram shows the concentration of secreted NTs  $\pm$  SD in each sample. The amount of secreted NTs was significantly higher in the supernatants of activated anti-MBP T cells that were stimulated with ConA or with their specific antigen MBP than in anti-MBP T cells that were incubated with the non-specific antigen OVA or with no antigen ( $p < 0.01$  or  $p < 0.001$  for all NTs, one-way ANOVA followed by Bonferroni's multiple comparison t-test). There were no significant differences in amounts of secreted NTs between the anti-MBP T cells that were incubated with OVA and with no antigen.

**Figure 6.** Expression of mRNA for TrkA, TrkB and p75 receptors in the rat optic nerve. Total RNA was extracted from normal and injured optic nerves, and qualitative RT-PCR was performed using primers specific to the various NT receptors. Normal (N) and injured (I) optic nerves expressed mRNA for TrkA, TrkB and p75 receptors, but not for TrkC receptor.

**Figure 7.** K252a inhibits the neuroprotective effect of anti-MBP T cells. **A.** The course and severity of EAE are not influenced by local treatment with K252 eye drops. Immediately after moderate optic nerve injury Lewis rats were injected i.p. with anti-MBP T cells, and then treated three times a day, from day 1 to day 14, with 1  $\mu$ M of K252a, K252b, or vehicle eye drops. EAE was evaluated according to a neurological paralysis scale. Data points represent means  $\pm$  SE. The figure shows the result of a representative experiment, in which each group contained five rats. No difference in the course of EAE or in disease severity was observed among the groups. **B.** The mean number of surviving RGCs is significantly diminished by treatment with K252a eye drops. Immediately after moderate optic nerve injury, Lewis rats were injected i.p. with anti-MBP T cells or with PBS and then treated three times a day, from day 1 to day 14, with 1  $\mu$ M of K252a, K252b, or vehicle eye drops. On day 14, 4-Di-10-Asp was applied to the optic nerves. After 5 days the retinas were excised and flat-mounted. Labeled RGCs from five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy, and their average numbers per mm<sup>2</sup> were calculated. The histogram shows the mean number of RGCs  $\pm$  SE. Each group contained five to ten rats. The neuroprotective effect of the anti-MBP T cells compared with that of PBS in the rats treated with vehicle eye drops was significant ( $p < 0.001$ , one-way ANOVA followed by Bonferroni's multiple comparison t-test). The neuroprotective effect of the anti-MBP T cells was significantly decreased in rats treated with K252a eye drops relative to rats treated with

vehicle eye drops ( $p < 0.05$ , one-way ANOVA followed by Bonferroni's multiple comparison t-test). The neuroprotective effect of the anti-MBP T cells in rats treated with K252b eye drops did not differ significantly from that in rats treated with vehicle eye drops. No effect of K252a or K252b eye drops on RGC survival was observed in the control PBS-injected rats.

**Table 1.** Details of primers used in the study.

Gene	Product Size (bp)	Direction	Sequence
IL-4	294	Forward (F)	TGCTTTCTCATATGTACCGGG
		Reverse (R)	TGAGTTCAGACCGCTGACAC
IL-6	371	F	ACTGCCTTCCCTACTTCAC
		R	GTATTGCTCTGAATGACTCTG
IL-10	329	F	GAGTGAAGACCAGCAAAGGC
		R	TCGCAGCTGTATCCAGAGG
TGF- $\beta$	411	F	AAGGAGACGGAATACAGG
		R	TGTGTTGGTTGTAGAGGG
IFN- $\gamma$	405	F	ATGAGTGCTACACGCCGCGTCTTGG
		R	GAGTTCATTGACTTTGTGCTGG
NGF	271	F	TGCTGAACCAATAGCTGCC
		R	ATCTCCAACCCACACACTGAC
BDNF	574	F	GCTGACACTTTTGAGCAC
		R	AAATCCACTATCTTCCCC
NT-3	510	F	CTCTCTCAATTCCCTCATTATC
		R	GCTTCTTTACACCTCGTTTC
NT-4/5	580	F	CCCTGCGTCAGTACTTCTTC
		R	TTTCCTCGTCTCTCTTGCC
TrkA	571	F	CACTAACAGCACATCAAGAGAC
		R	GAAGACCATGAGCAATGGG
TrkB	188	F	TCATTGGGATGACCAAGATCC
		R	GCAGAGGTTATAGCACTCC
TrkC	503	F	CTTAATATTTCAGTCGCTGTTGTGG
		R	TGATCCTTGTGGATGGACAGCCAC
P75	447	F	TGCAGTGTGCAGATGTGCCTATGGC
		R	AGGAATGAGCTTGTCGGTGGTGCCG

Fig. 1

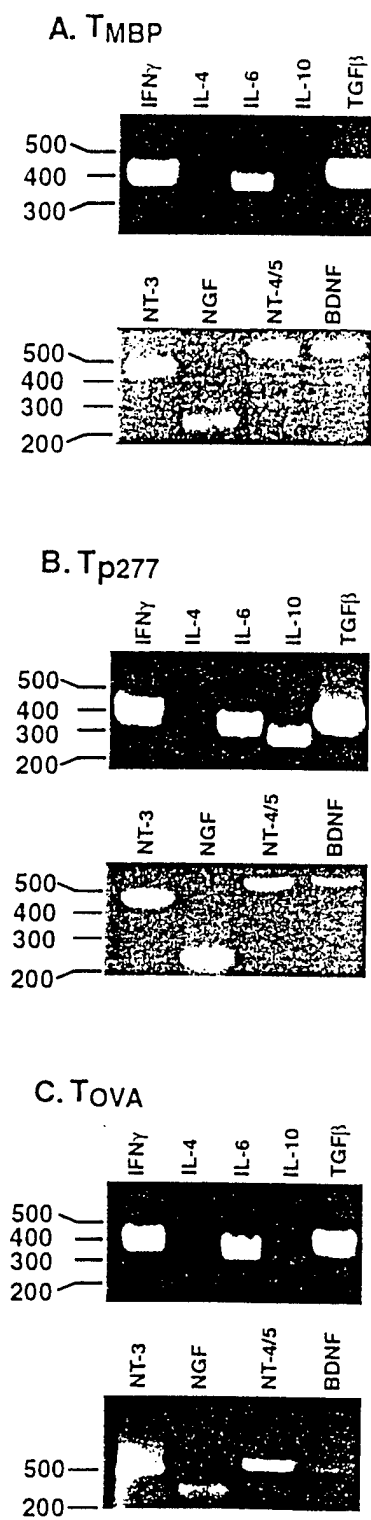


Fig. 2

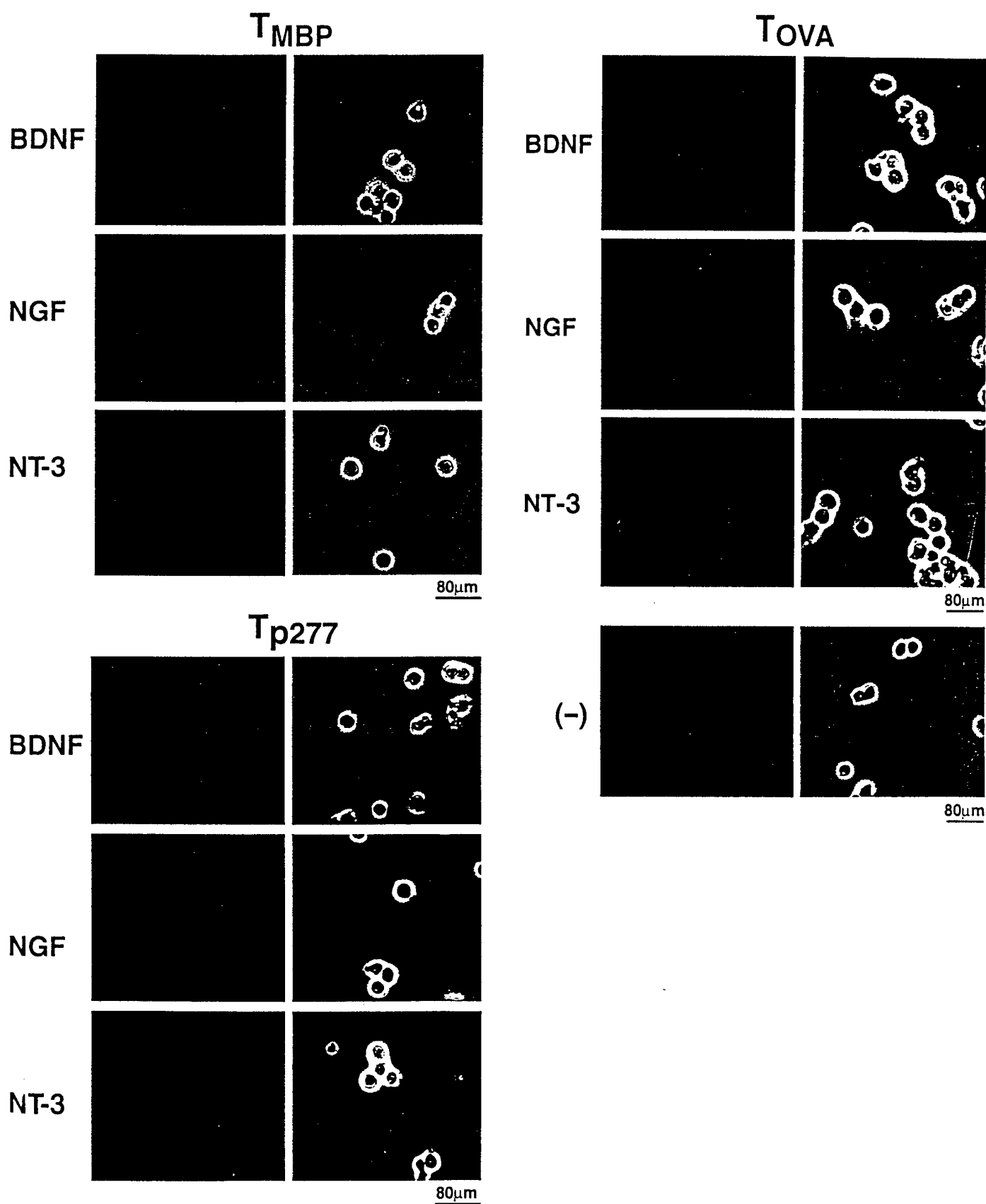


Fig. 3.

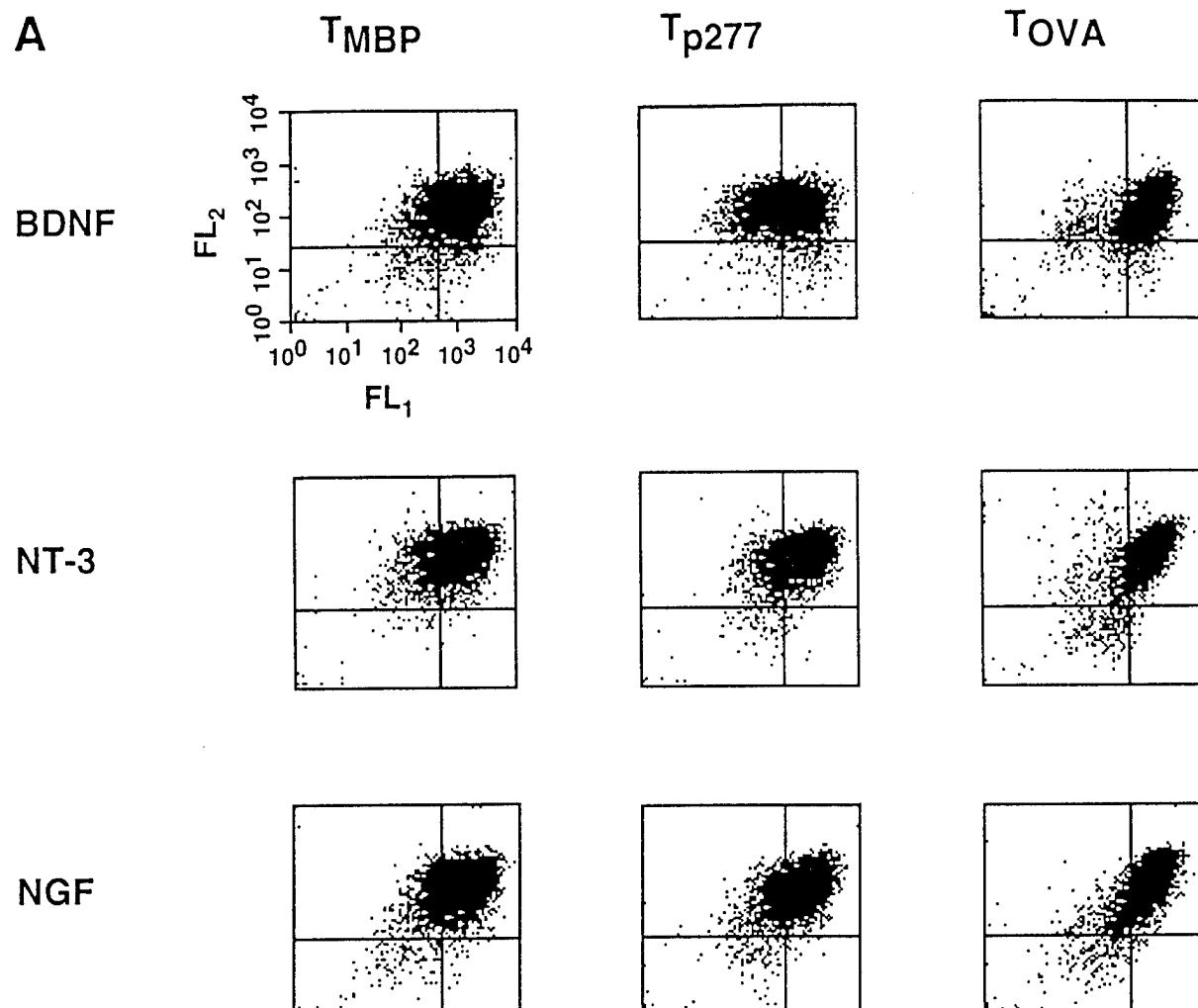
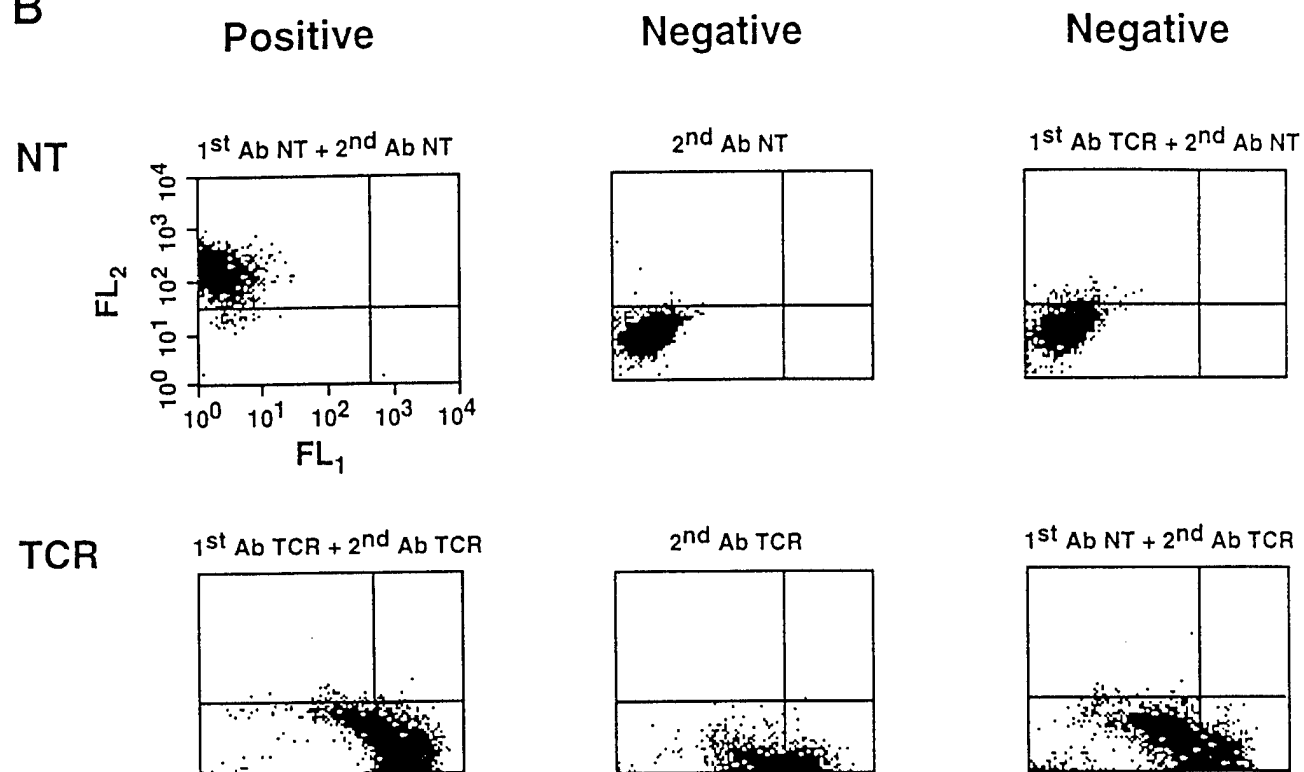


Fig. 3

B



Histogram of TCR populations

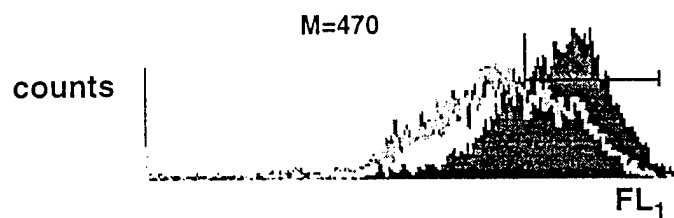


Fig. 4

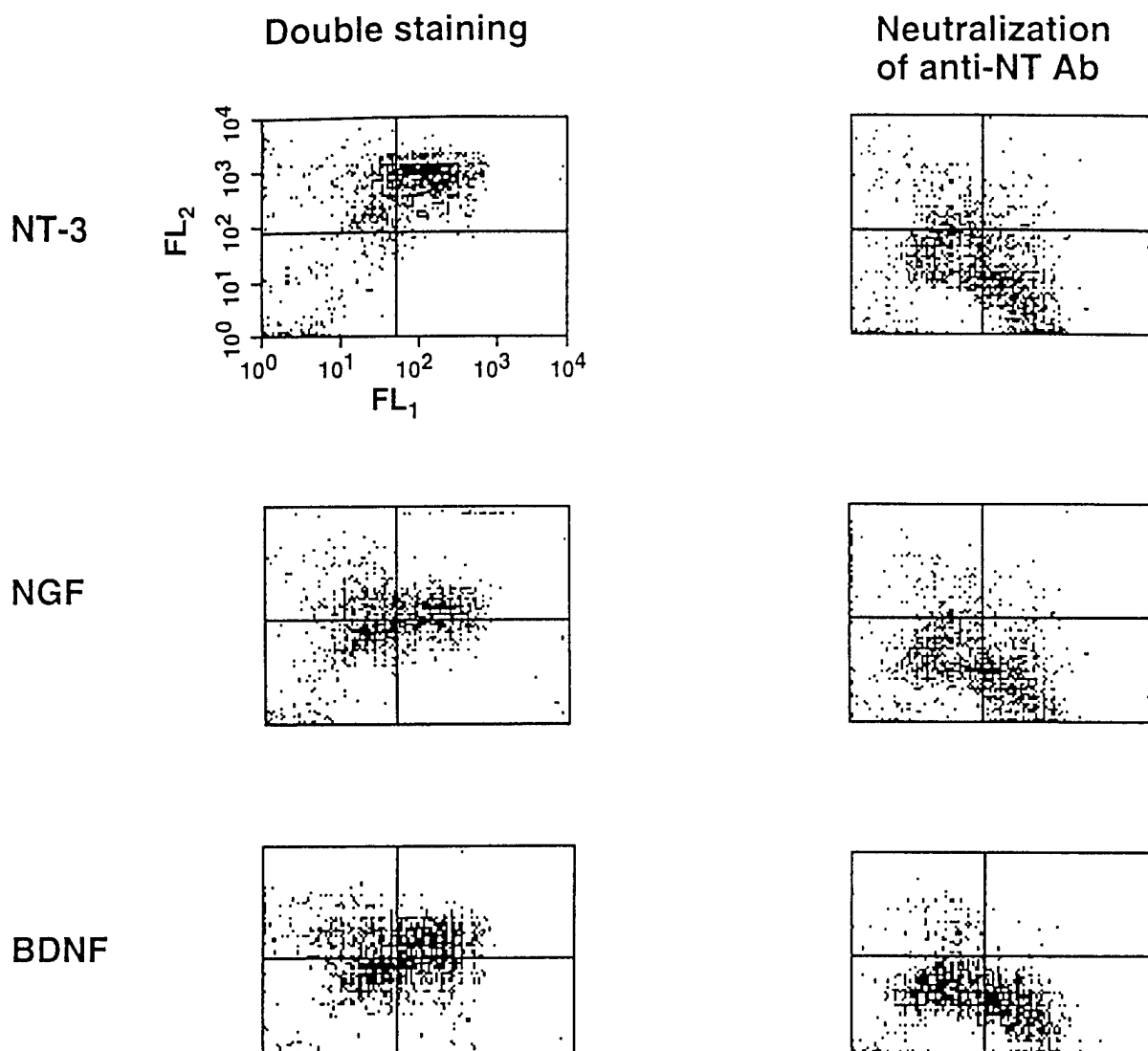


Fig. 5

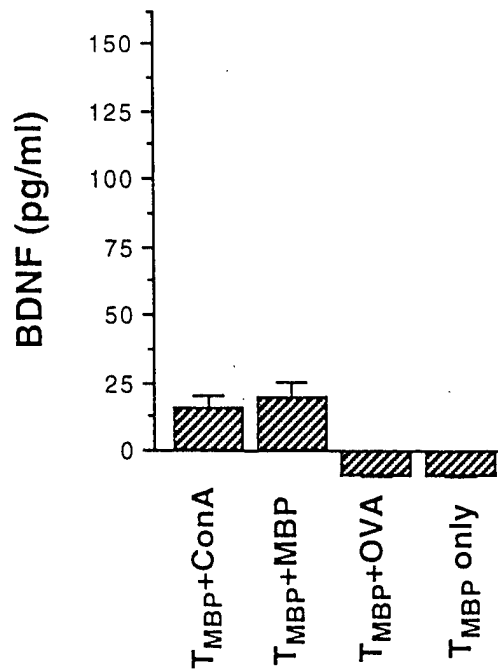
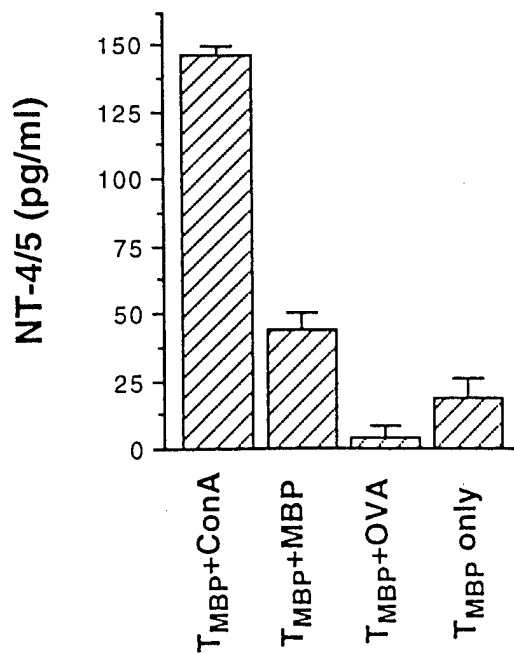
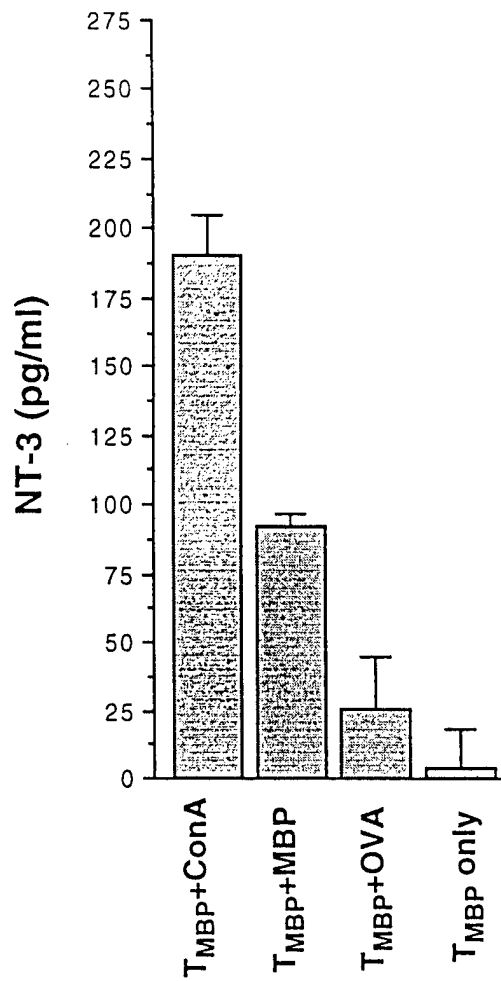
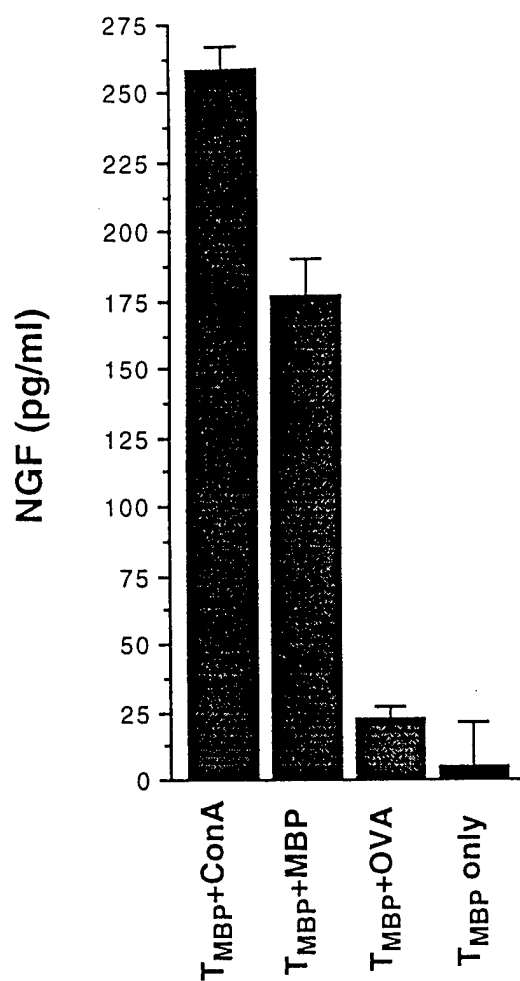


Fig. 6

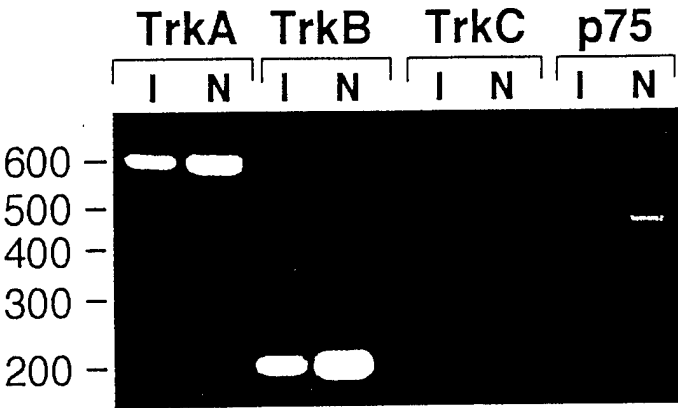
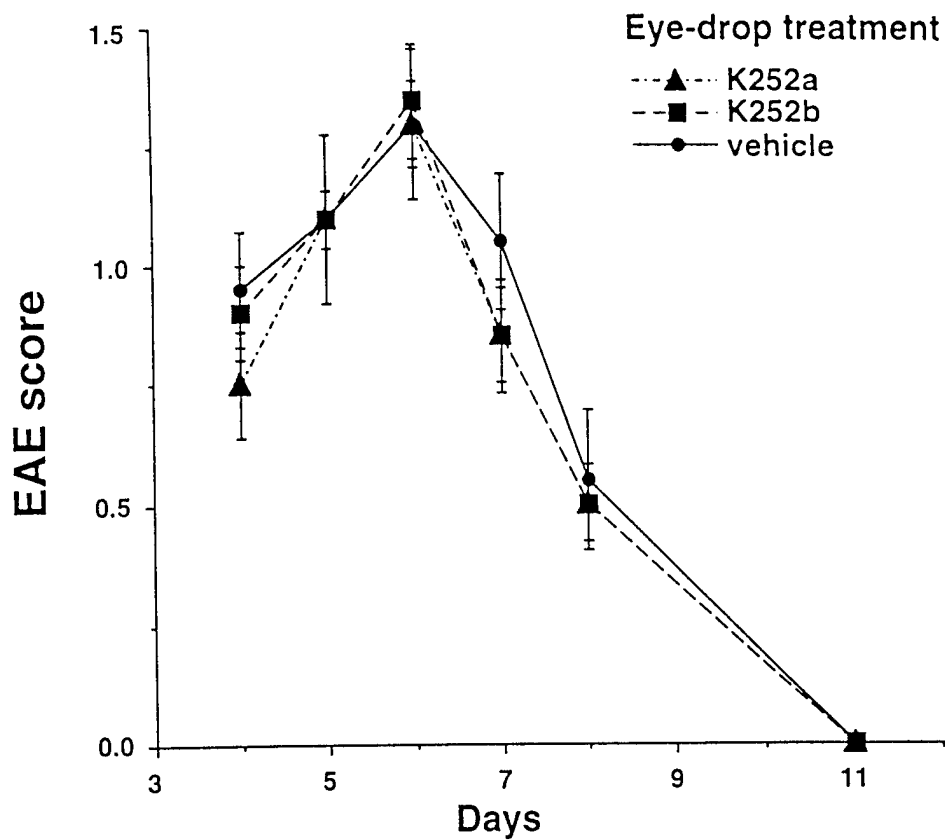


Fig. 7

**A**



**B**

